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Selecting Drought Tolerant Soybean Genotypes Using QTLs Associated with Shoot Ureide and Nitrogen Concentrations

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ABSTRACT

In soybean, nitrogen fixation is more sensitive to drought than other physiological processes like photosynthesis. The sensitivity of nitrogen fixation to drought has been associated with high shoot concentrations of ureide and nitrogen under well-watered conditions. Previous research by Hwang et al. (2013) detected quantitative trait loci (QTLs) in a KS4895 by Jackson population associated with shoot ureide and nitrogen concentrations. The present research evaluated the use of these QTLs in selecting genotypes with drought tolerant nitrogen fixation. Our objectives were to compare actual versus expected phenotype of recombinant inbreed lines (RILs) selected using molecular markers, and to evaluate the effects of shoot nitrogen and ureide concentrations on nitrogen fixation and yield under well-watered and drought conditions. We also evaluated differences in ureide concentration in four near-isogenic line sets that were developed based upon preliminary QTL data for ureide concentration. Isolines did not differ in ureide concentration, and subsequently we determined that preliminary QTLs were not associated with shoot ureide concentration. In 2011, field experiments were conducted in Fayetteville using 12 RILs selected using preliminary QTLs. Selection based on preliminary QTL information did not result in the expected phenotypes for ureide and nitrogen concentrations. Under severe drought conditions, however, RILs with low well-watered ureide and nitrogen concentrations had an increase in growth rate, nitrogen fixation rate, and yield (r^2>0.50, P<0.001). In Fayetteville and Keiser 2012 field experiments, RILs were selected using QTL detected by Hwang et al. (2013). Selection resulted in the expected phenotypes for ureide and nitrogen concentrations. Under well watered conditions, genotypes with alleles for high ureide and nitrogen concentrations showed higher nitrogen fixation rates, higher percentages of nitrogen derived from the atmosphere and higher yields than genotypes with alleles for low ureide and nitrogen concentrations (r^2>0.20, P<0.0001). Since nitrogen concentration may be

positively correlated with yield under well watered conditions, genotypes with high yield and low shoot ureide and nitrogen concentrations need to be identified. The QTLs detected by Hwang et al. (2013) are an important tool for identifying these genotypes.

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Finally, I would like to extend my gratitude to everybody in the Soybean Physiology lab, and to my friends and family.

DEDICATION

This thesis is dedicated to my wife Sofía Rojo, my brother Patricio Bolton, my sister María Teresa Bolton, and my parents Alejandro Bolton and María Teresa Campastro.

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CHAPTER I

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] was first domesticated in China from the 11th to the 7th century B.C.E (Hymowitz and Singh, 1987), and was introduced to the United States (U.S.) in the 1700's (Hymowitz and Harlan, 1983). At present, soybean is the most cultivated oilseed crop in the world. It is grown in about 50 countries, with the U.S. leading the world's production during the past half century. Soybean is the second largest field crop in the U.S. and was planted on 30.35 million hectares in 2011 with a production of about 83.28 million tons, representing 33% of total world soybean production (USDA, 2012). In Arkansas, soybean is one of the most important field crops, and in 2011 was grown on a total area of 1.34 million hectares, with a total production of 3.38 million tonnes (USDA, 2012).

Nitrogen

Nitrogen is an essential mineral element involved in several metabolic processes in plants. Nitrogen is a component of proteins, nucleic acids and the energy-rich molecule adenosine triphosphate (ATP) which provides energy for many biochemical reactions. Moreover, nitrogen is a central component of two molecules involved in photosynthesis: chlorophyll and ribulose 1,5-bisphosphate carboxylase oxigenase (RuBisCo). Therefore, sufficient nitrogen is critical for photosynthesis (Wittenbach et al., 1980).

Soybean requires a high amount of nitrogen to synthesize leaf proteins and seed storage globulins and glycoproteins. Compared with other row crops, soybean has the greatest nitrogen demand per unit of seed (Sinclair and de Wit, 1976). This high requirement makes nitrogen availability an important factor affecting seed yield. Because soybean is not typically fertilized with nitrogen, seed production and seed yield depend greatly upon biological nitrogen fixation (BNF).

Biological nitrogen fixation

Soybean is a leguminous crop, which has the ability to form a symbiotic relationship with the nitrogen fixing bacteria *Bradyrhizobium japonicum*. Soybean produces root nodules as a result of the infection of the root hairs by the symbiotic bacteria. Bacteroids infect cells in the central region of the nodule. This central region is enclosed by the nodule cortex, which is thought to act as a gas diffusion barrier which regulates gas exchange in the nodule (Hunt and Layzell, 1993). In this central region of the nodule biological nitrogen fixation takes place, in which atmospheric nitrogen (N_2) is reduced to ammonium (NH_4+) by the nitrogenase enzyme that is produced by the bacteroid. The overall reaction can be represented by the following equation:

$$N_2 + 8e^- + 10H^+ + 16\ ATP \longrightarrow 2NH_4^+ + 16ADP + 16Pi + H_2 \qquad [1]$$

Following BNF, in the infected plant cytosol cells surrounding the bacteroid, ammonium is incorporated into glutamine by glutamine synthetase (GS, E.C.6.3.1.2). Then, in the proplastids, glutamine is converted to glutamate by glutamate 2-oxo-glutarate amino transferase (GOGAT, E.C.1.4.1.14, Newcomb and Tandon, 1981; Goodwin and Mercer, 1983). Glutamate is subsequently assimilated to purines. By partial oxidation of purines, uric acid is formed in the cytosol of infected cells and transported to uninfected cells. There, it is oxidized to allantoin in peroxisomes by uricase (E.C.1.7.3.3) and finally, in the endoplasmic reticulum, converted to allantoic acid by allantoinase (E.C.3.5.2.5, Boland et al., 1982). The ureides, allantoic acid and allantoin, are then exported to the shoots via the xylem and transported to the rest of the plant via the transpiration stream (Walsh, 1995).

Ureide breakdown

In the shoot, allantoinase transforms allantoin to allantoate (E.C. 3.5.2.5). Allantoate is

then catabolized to ureidoglycolate by two possible pathways, both of which have been reported in soybean. In the first pathway, allantoate is converted to urea and ureidoglycolate by allantoate amidinohydrolase (E.C. 3.5.3.4). And then urea is catabolized to $2NH_4^+$ and CO_2 by urease (E.C. 3.5.1.5). In the second pathway, allantoate is converted to ureidoglycine, CO_2 and, NH_4^+ by allantoate amidohydrolase (E.C. 3.5.3.9) which requires manganese (Mn^{+2}) as a co-factor (Lukaszewski et al., 1992). Subsequently, ureidoglycine is converted to ureidoglycolate and NH_4^+ (Winkler et al., 1987). Ureidoglycolate catabolism has two possible pathways as well, but just one has been reported in soybean (Purcell, 2009). In the pathway reported in soybean ureidoglycolate is converted to glyxolate, $2NH_4^+$ and CO_2 by ureidoglycolate amidohydrolase (E.C. 3.5.3.19). In the other pathway, reported in chickpea (Muñoz et al. 2001), ureidoglycolate urea-lyase, a manganese dependant enzyme, converts ureidoglycolate to urea, and glyoxylate. Finally, urea is catabolized to $2NH_4^+$ and CO_2 by urease (E.C. 3.5.1.5). Plants use NH_4^+ as a source of nitrogen and to construct amino acids.

Drought stress

Drought is the major limitation to crop production world-wide (Boyer, 1982). In the US, according to the National Oceanic and Atmospheric Administration, in the period 1950-2011, 15% of the soybean production was affected by water stress (NOAA, 2012). Irrigation may be a solution to alleviate water stress. However, given that less than 16% of the cultivated area has irrigation (FAO, 1991) this will only affect a small portion of soybean produced. Additionally, irrigation is expensive and requires fresh water, which is a scarce resource with an increasing competition among the recreational, urban, and agricultural uses. This competition will increase based on the United Nations (2006) that predicts that in 2025 one third of the world's population will live in water-stressed countries.

Strategies for ameliorating drought effects

Given that an increase in the area under irrigation is not feasible, there is the need to use other strategies for ameliorating drought effects. The strategies used can be divided into two mechanisms: drought avoidance and drought tolerance (Levitt, 1980). Drought avoidance means to grow a crop in a specific period when water is plentiful, whereas drought tolerance refers to adaptations that reduce water stress under drought conditions.

Drought avoidance can be implemented in regions that have a predictable dry period by growing a crop prior to the dry period. An example is the early season production system used in the southern Mississippi Delta region (Heatherly, 1999). In this system, early maturity soybean genotypes are sown in early spring when the soil is moist, and harvested in late July before the predictable dry period (Bowers, 1995; Purcell et al., 2003).

Drought tolerance mechanisms have been difficult to discover and implement. Usually drought tolerant plant species have traits that allow them to survive in dry conditions, but since crops are grown to produce abundant grain not just survive, traits associated with survival are not useful for improving crop's yields under drought. However, a few traits have been identified and proved to be successful in providing some drought tolerance (Sadok and Sinclair, 2011; Purcell and Specht, 2004). The traits can be grouped in three mechanisms described by Sadok and Sinclair (2011). These are: i) conserving soil water, ii) improving the access to more water, and iii) overcoming special water deficit sensitivities. The following section will describe three examples of these mechanisms, with the last example of nitrogen fixation being the focus of this research.

Conserving soil water by reducing transpiration has proven to increase yield under drought (Gillen and Shelton, 2006, 2007, 2008). A reduction of transpiration was obtained by a

slow-wilting trait which was found to decrease stomata conductance at high vapor pressure deficit (Fletcher et al., 2007, Sinclair et al., 2008). However, less transpiration means slower growth rate (deWit, 1958; Fischer and Turner, 1978; Tanner and Sinclair, 1983) and when water is plentiful slow-wilting genotypes may grow slower and yield less than fast-wilting genotypes.

Improving access to more water by increasing rooting depth may increase drought tolerance. Deeper roots may allow the crop access to water stored deep in the soil profile. By using a mechanistic soybean growth and yield model, Sinclair et al. (2010) simulated that if the rate in the depth of soil water extraction increased more water could be accessed by the crop and yield gains would occur. However, using deep water early in the season may make the crop more vulnerable to drought later in the season.

In soybean, nitrogen fixation is more sensitive to drought than other physiological processes such as photosynthesis and transpiration (Kuo and Boersma, 1971; Sinclair, 1986; Durand et al. 1987). In this regard, Sprent (1971) showed that the cortex of soybean root nodules can be damaged even with relatively mild water stress. Likewise, Durand et al. (1987) compared nitrogenase activity and photosynthesis during initial stages of drought and reported that nitrogenase activity declined 70% whereas photosynthesis declined only by 5%. Purcell and King (1996) demonstrated an increase in soybean biomass and nitrogen accumulation rate during drought in nitrogen-fertilized plants over those solely dependent on BNF. Several other studies also confirm the sensitivity of the BNF to drought (Sinclair et al., 1987; Djekoun and Planchon, 1991; Sall and Sinclair, 1991). Drought-tolerant nitrogen fixation may provide soybean with some drought tolerance. Sinclair et al. (2010) in a simulation of the benefits of altered soybean drought traits identified drought-tolerant nitrogen fixation as the most beneficial trait with yield increases on both dry and wet years.

Inhibition of nitrogen fixation by accumulation of ureides

The physiological basis of the inhibition of BNF by water stress in soybean nodules is not clearly understood. A possible explanation for the decrease in nitrogenase activity under drought is a feedback mechanism involving the accumulation of ureides in leaves at initial stages of water stress (Sinclair and Serraj, 1995; Serraj et al., 1999; Vadez et al., 2000), which may be facilitated by a decreased ureide catabolism and reduced phloem export under water stress (Vadez et al., 2000). Additionally, artificial application of ureides inhibits nitrogenase activity, demonstrating the possibility of this mechanism (Serraj et al., 1999; King and Purcell, 2005).

It has been shown that ureide concentration varies among genotypes. Since ureides accumulation possibly triggers the feedback inhibition mechanism, genotypes with low ureide accumulation may be able to prolong nitrogen fixation under water stress compared with genotypes with high ureide concentration. Purcell et al. (1997), in a growth chamber experiment, compared Jackson, a low-ureide and low-yielding cultivar released in 1953 (PI 548657, Johnson, 1958), with KS4895, a high-ureide and high-yielding cultivar released in 1998 (PI 595081, Schapaugh and Dille, 1998). They found that nitrogen fixation under water deficit as measured by the acetylene reduction assay was decreased at a higher soil-moisture level in KS4895 than in Jackson.

Nitrogen and ureide concentration

More recent research has shown that well watered shoot nitrogen and ureide concentration is related with nitrogen fixation ability under water stress (King and Purcell, 2006; King et al., 2013). King and Purcell (2006) in a greenhouse and field experiments found that shoot nitrogen concentration decreased in response to water deficit in genotypes with high nitrogen concentration under well watered conditions, however, it was not reduced in genotypes

with low nitrogen concentration. King et al. (2013) in a growth chamber experiment measuring nitrogen fixation by the acetylene reduction assay (ARA) in twelve plant introductions with extreme values of shoot nitrogen concentration, showed that genotypic differences for sensitivity of nitrogen fixation to water stress was correlated with shoot ureide and nitrogen concentration under well-watered conditions and shoot ureide concentration under drought conditions. King et al. (2013) concluded that shoot nitrogen concentration under well-watered conditions can be useful for identifying genotypes with drought tolerant nitrogen fixation. Conversely, since shoot ureide concentration and shoot nitrogen concentration are highly correlated (King and Purcell, 2006; Hwang et al. 2013; King et al., 2013) selection based on either of the traits would likely pick similar genotypes.

Shoot ureide concentration trait

Sall and Sinclair (1991) investigating the genetic variation of soybean cultivars in their ability to fix nitrogen under water stress identified Jackson as a cultivar with nitrogen fixation tolerable to drought. The ability of Jackson to continue nitrogen fixation under drought was then confirmed in greenhouse (Sall and Sinclair, 1991; Serraj and Sinclair, 1996; Purcell et al., 1997, 2000) and field experiments (Serraj and Sinclair, 1997).

In 1993, Jackson was crossed with several high yielding cultivars, including KS4895. KS4895 is a cultivar released by the Kansas State University (Schapaugh and Dille, 1998); it is a widely adapted, high yielding cultivar. Conversely, KS4895 is a cultivar with nitrogen fixation sensitive to drought (Purcell et al., 1997, 2000). A plant population of recombinant-inbred lines (RILs) composed of F_3- and F_5- derived lines was developed by single-seed descent from the KS4895 by Jackson cross (Charlson et al., 2009). Seed were bulked from F_1 plants and the resulting F_2 plants were advanced by single seed descent to the F_3 or F_5 generations. Seed from

the individual F_3 or F_5 were bulked to originate F_3- or F_5-derived RILs.

Since KS4895 is maturity group IV and Jackson is maturity group VII the RILs produced of this hybridization segregated for maturity. One hundred $F_{3:4}$ lines of maturity group V were selected and subjected to further evaluations (Sinclair et al., 2007). From that one hundred $F_{3:4}$ lines, 17 lines were selected based on nitrogen accumulation under water stress in a greenhouse experiment. These 17 lines were subsequently tested in water limited environments, and two lines showed yield greater than commercial checks. These two lines were then tested for drought tolerant nitrogen fixation by the acetylene reduction assay (ARA) method in a greenhouse experiment. Both lines showed greater nitrogen fixation under water stress than their sensitive parent. These lines were released to the public and are a valuable genetic resource for environments with moderate water stress (Sinclair et al., 2007, 2010).

Quantitative Trait Locus

A quantitative trait locus (QTL) is a region of the chromosome where the genetic information of a quantitative trait is located. These regions are identified using molecular markers. Molecular markers do not represent the target genes themselves but act as 'tags' due to their proximity to the particular genes. They are often located in non-coding regions of the DNA and consequently do not affect the gene expression. Association of molecular markers with phenotypic traits can subsequently be used as selection criteria in marker assisted selection (Collard et al., 2005).

Using 97 RILs from the previously described population of KS4895 and Jackson, QTLs for shoot N concentration and shoot ureide concentration under well watered conditions were identified (Hwang et al., 2013). Composite interval mapping (CIM) identified five QTLs for ureide concentration and four for nitrogen concentration. Multiple interval mapping (MIM)

identified two QTLs for ureide concentration and one for nitrogen concentration all with similar locations as that identified with CIM (Hwang et al., 2013).

In our research program, we confirmed these QTLs and analyzed its relationship with nitrogen fixation and yield under well watered and drought conditions. In subsequent chapters the relationship between these QTL, shoot ureide concentration, shoot nitrogen concentration, grain yield and nitrogen fixation under well watered and drought conditions are examined in detail.

The hypothesis of the present research was that by using molecular marker assisted selection we could select genotypes with alleles that would result in low shoot ureide concentration and low nitrogen concentration, and that those genotypes would have superior yield under drought because of high nitrogen fixation rates. In chapter 2 we compared ureide concentration of near-isogenic lines with contrasting alleles for shoot ureide concentration. In chapter 3 we selected genotypes using QTLs associated with shoot ureide and nitrogen concentration and we evaluated the ability of the QTLs to predict phenotype, yield, and nitrogen fixation.

CHAPTER II

UREIDE CONCENTRATION OF NEAR-ISOGENIC LINES WITH CONTRASTING ALLELES FOR SHOOT UREIDE CONCENTRATION

ABSTRACT

In soybean nitrogen fixation is more sensitive to drought than other physiological processes such as photosynthesis. The physiological basis of the inhibition of nitrogen fixation under water stress is not clearly understood. Nevertheless, there is an association among nitrogen fixation, shoot ureide concentration, and shoot nitrogen concentrations. Jackson was identified as a cultivar with drought tolerant nitrogen fixation, and it was crossed with drought sensitive KS4895. Parents differed in shoot ureide concentration, nitrogen concentration and nitrogen fixation ability under drought. Recombinant inbreed lines were phenotyped for shoot ureide and nitrogen concentration and genotyped using 195 polymorphic simple sequence repeat markers (SSRs). Preliminary quantitative trait loci (QTLs) associated with shoot ureide concentration were detected using single point analysis. Four sets of near-isogenic lines (NILs) were created by selfing inbred lines that segregated for the QTLs of interest. Within a NIL set, alleles should be almost identical except for the QTLs associated with shoot ureide concentration. A growth chamber experiment was conducted to determine if there was variation in the ureide concentration between lines in each NIL set under well-watered and drought conditions. Stem, nodule, and total plant ureide concentration ($\mu mol\ gdw^{-1}$) increased under drought. Only one NIL set showed differences in plant ureide concentration ($\mu mol\ gdw^{-1}$). A more thorough QTL analysis done by Hwang et al. (2013) determined that these preliminary QTLs were not associated with shoot ureide concentration, and this explains the absence of differences between lines within most NIL sets.

Introduction

Drought is the most important limitation to crop production world-wide (Boyer, 1982). In Arkansas, although the majority of soybean is grown with irrigation (NASS, 2012) frequent high evaporative demand and insufficient rainfall during the season may reduce yield if soybean is not irrigated properly.

In soybean, nitrogen fixation is very sensitive to drought (Sprent, 1971; Kuo and Boersma, 1971; Durand et al., 1987; Djekoun and Planchon, 1991; Sall and Sinclair, 1991). In this regard, Purcell and King (1996) showed that under drought, nitrogen availability limited yield. Consequently, drought tolerant nitrogen fixation may increase yield under drought.

The physiological basis of the inhibition of nitrogen fixation by water stress is not clearly understood. In early research it was hypothesized that under drought, shoot ureide accumulation triggered a feedback mechanism inhibiting nitrogen fixation in the nodules (Purcell et al., 2000). More recent studies showed that shoot ureides were not directly involved in the inhibition of nitrogen fixation under drought (King and Purcell, 2005; Ladrera et al. 2007). Although shoot ureides are not directly involved in the inhibition of nitrogen fixation, there is a loose association among shoot ureide concentration, shoot nitrogen concentration and drought tolerant nitrogen fixation (King and Purcell, 2006). King et al. (2013) demonstrated that genotypes with low ureide and nitrogen concentrations continued nitrogen fixation at dryer soil conditions than genotypes with high ureide and nitrogen concentrations.

Sall and Sinclair (1991) identified Jackson as the cultivar with least sensitivity to drought among a group of 8 genotypes previously selected for drought tolerance. The drought tolerant nitrogen fixation of Jackson was later confirmed in greenhouse (Sall and Siclair, 1991; Serraj and Sinclair, 1996; Purcell et al., 1997, 2000) and field experiments (Serraj and Sinclair, 1997).

A population was created in 1993 by crossing KS4895 with Jackson (Charlson, et al. 2009). KS4895 is a high yielding cultivar with high shoot ureide concentration, high nitrogen concentration, and drought sensitive nitrogen fixation. Conversely, Jackson has low shoot ureide and nitrogen concentrations and drought tolerant nitrogen fixation (Purcell et al., 1997; King and Purcell, 2006). In preliminary mapping, quantitative trait loci (QTLs) were associated with shoot ureide concentration, and several recombinant inbred lines (RILs) were identified that were heterozygous at the loci associated with ureide concentration. Near-isogenic lines (NILs) were created by selfing RILs that were heterozygous at the loci of interest as described by Tunistra et al. (1997). At the F_5 generation it is expected that 93.75% of the alleles would be identical from plants within a RIL. The hypothesis of this research was that NILs with different alleles associated with shoot ureide concentration would result in contrasting phenotypes. The objective of the following experiment was to determine whether ureide concentration was different between lines of several NIL sets.

Materials and methods

Population development

Ninety two recombinant inbred lines (RILs) were created by crossing cultivars KS 4895 and Jackson and selfing the progenies to the F_5 generation, at which point genotypes are expected to be 93.75% homozygous (Charlson et al., 2009). The RILs were then phenotyped for quantitative traits including shoot ureide, nitrogen, Mn, and genotyped using 195 polymorphic simple sequence repeat markers (SSRs). An analysis of variance performed on the phenotypic data showed that the phenotypic traits for ureide concentration were associated with specific SSRs. After identifying the loci significantly associated with ureide concentration, individual RILs were evaluated for zygosity. Those lines that were heterozygous for the loci associated with ureides were chosen for subsequent selfing to produce homozygous lines that were contrasting for the loci of interest (known as near-isolines, NILs) (Tunistra et al., 1997). A NIL set consists of the homozygous lines that are almost identical except for the loci of interest (Table 2.1), and within an NIL set, the number of polymorphic loci should be the same as that expected from an F_5 population (6.25%).

Growth chamber experiment

In summer 2010, a pot culture experiment was conducted in a growth chamber to determine if there was any difference in ureide concentration ($\mu mol\ g^{-1}$) between lines within NIL sets. Based upon the molecular marker data, we made an *a priori* assumption of the ureide concentration ($\mu mol\ g^{-1}$) (referred to as "expected" in statistical evaluation). The study included well-watered (WW) and drought-stressed (DR) treatments, and the experimental design was a randomized complete block design (RCBD) with four replications. Two seeds were planted in each plastic pot (15 cm dia x 12 cm deep) containing a 4:1 soil mixture of LB2 potting soil (Sun

Table 2.1. Near-isogenic lines (NILs) used in the 2010 growth chamber experiment.

NIL set	Line	SSR loci		Expected ureide concentration
		Satt220	Satt306	
8	1	K†	K	High
8	2	K	J‡	Low
9	1	J	J	Low
9	2	J	K	High
10	1	K	J	Low
10	2	J	K	High
11	1	K	J	Low
11	2	K	K	High

† K= allele from KS 4895

‡ J=allele from Jackson.

Gro Horticulture Co. Canada Ltd.) and Captina silt loam soil. The pots were fertilized with 515 ml per pot of –N Hoagland's solution, and the seeds were treated with metalaxyl (Ridomil Gold®, Syngenta) to prevent seedling diseases. The pots were then inoculated with *Bradyrhizobium japonicum* (strain USDA 110). The saturated weight of the pots was measured after watering the pots to saturation and letting them drain overnight. The seedlings were thinned to one plant per pot at the V1 stage (Fehr and Caviness, 1977). Evaporation was minimized by covering the surface of the pots with plastic bags. The growth chamber was maintained at a constant day and night temperature of 25°C. Relative humidity was approximately 40% during day and 20% at night. Approximately 600 μmol m^{-2} s^{-1} of photosynthetically active radiation at the top of the canopy was supplied for a 16 hour photoperiod.

All the pots were maintained between 60 and 70% of the saturated weight until V6 when the DR treatment was initiated. Well watered plants were kept at 65% saturated weight while DR plants were not watered until day 4 when a small amount of water was added. Relative transpiration was normalized for controlling variation between plants and days using the procedure described by Ray and Sinclair (1997). Drying was allowed in the DR treatment plants until the relative transpiration decreased to 0.6 at day 6. At which point the DR plants were under water stress and were harvested. After harvest, the plant parts were separated into shoots, leaves, roots, and nodules and dried at 40°C. After drying, the samples were weighed and ground. Subsamples of 125 mg of roots and stems, 9 mg of leaves, and 25 mg of nodules were used to determine ureide concentration using the colorimetric procedure of Young and Conway (1942) as described by de Silva et al. (1996).

The SAS 9.2 (2008) statistical software package (SAS Institute Inc., Cary, NC, USA) system was used for analysis of variance (ANOVA), using a general linear model (PROC GLM),

and means were separated by Fisher's protected least significance difference (LSD, $P = 0.05$).

Results

Plants under well-watered conditions maintained the relative transpiration around 1 during the whole water treatment period (Figure 2.1). Plants in the drought treatment decreased their relative transpiration drastically after day 2, then increased slightly after re-watering at day 4, and were harvested at day 6, at which point were under water stress.

The analysis of variance showed that the interaction among the factors water treatment, NIL set and expected ureide concentration was only significant for the total ureide concentration per plant (Table 2.2), and therefore this variable was analyzed separately. The water treatment and NIL set interaction was significant for the variables stem and nodule. Near-isolines set effect was significant for root and total ureide concentration per plant. In the total ureide concentration variable water treatment was also significant.

Ureide concentrations measured in stem and nodules were significantly higher under drought than under well-watered conditions for most of the isolines except for the stem ureide concentration in the NIL set 8 (Table 2.3). Isoline set 8 had the highest root and total plant ureide concentration. Total plant ureide concentration was higher in the DR than in the WW treatment.

Total plant ureide content (μmol $plant^{-1}$) in the NIL sets 9, 10, and 11 did not differ from expected low or high ureide concentration for the lines investigated (Table 2.4). This indicates that selection for QTL in NIL sets 9, 10 and 11 did not result in different ureide concentration. NIL set 8 showed the expected response for total plant ureide concentration under well-watered conditions but no significant difference under drought conditions.

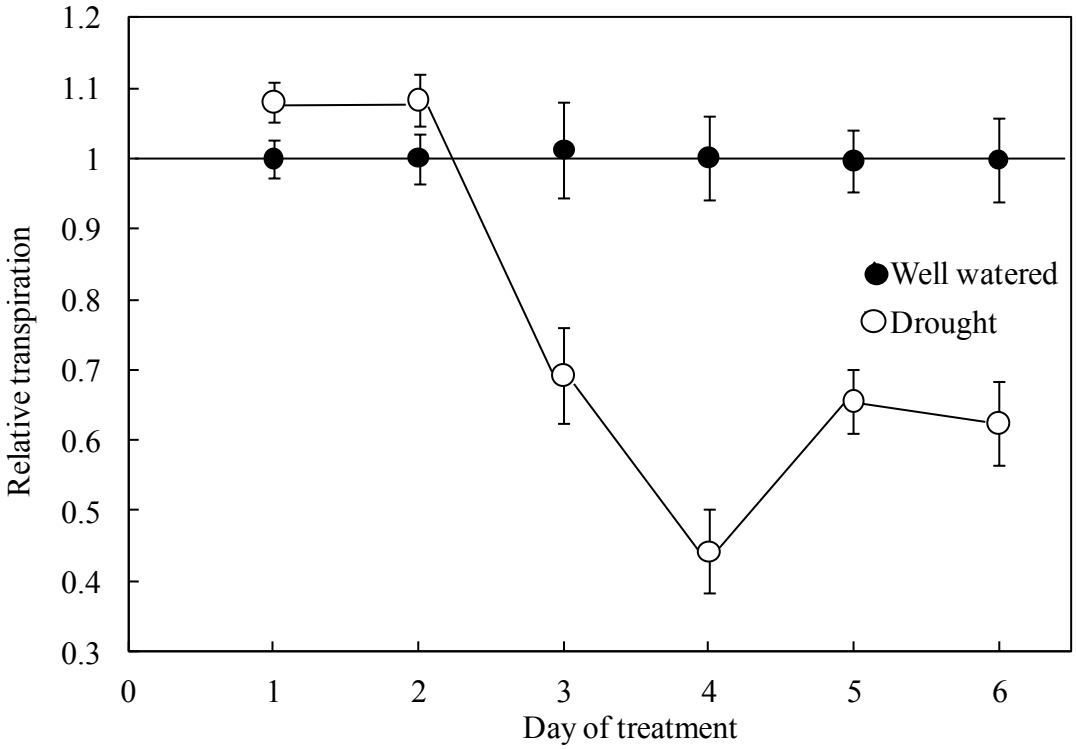

Figure 2.1. Relative transpiration in the well-watered and drought treatments in the 2010 growth chamber experiment. Bars represent standard errors averaged across genotypes.

Table 2.2. Analysis of variance for stem ureide concentration (stem, µmol gdw^{-1}), leaf ureide concentration (leaf, µmol gdw^{-1}), nodule ureide concentration (nodule, µmol gdw^{-1}), root ureide concentration (root, µmol gdw^{-1}), total ureide per plant (total_pl, µmol $plant^{-1}$), and total ureide concentration per plant (tot_conc, µmol gdw^{-1}) of soybean near isoline sets grown under well-watered or drought conditions for 10 days prior to harvest at V6 in the 2010 growth chamber experiment. Near-isolines were selected using preliminary QTLs associated with either high or low ureide concentrations (expected ureide concentration) from a recombinant inbreed population.

Source of variance	Stem	Leaf	Nodule	Root	Total_pl	Tot_conc
Water trt.[†]	**	ns	**	ns	ns	**
NIL set	**	ns	**	**	**	**
Expected [‡]	ns	ns	ns	ns	ns	ns
NIL set x expected	ns	ns	ns	ns	ns	ns
Water trt. x NIL set	*	ns	*	ns	ns	ns
Water trt. x expected	ns	ns	ns	ns	ns	ns
Water trt. x NIL set x expected	ns	ns	ns	ns	*	ns

[†]Water treatment.

[‡]Expected ureide concentration.

**Significant at the 0.01 probability level.

*Significant at the 0.05 probability level.

Ns = not significant at the 0.05 probability level.

Table 2.3. Ureide concentration (µmol gdw^{-1}) means of stem, nodule, root and total plant conentration (tot_conc) of soybean near isoline sets grown under well watered or water deficit conditions for 10 days prior to harvest at V6 in 2010 growth chamber experiment. Near-isolines were selected for alleles for either high or low ureide concentrations (expected ureide concentration) from a recombinant inbreed population.

NIL set	Water treatment	Stem	Nodule	Root	Tot_conc
8	DR	23.14 abc†	31.49 ab	-¶	-
8	WW	26.13 ab	21.32 c	-	-
9	DR	18.57 cd	21.01 c	-	-
9	WW	12.46 e	15.05 d	-	-
10	DR	21.25 bc	28.65 b	-	-
10	WW	13.65 de	18.38 cd	-	-
11	DR	27.24 a	34.93 a	-	-
11	WW	18.40 cd	15.02 d	-	-
8	avg‡	-	-	19.91a	10.97 a
9	avg	-	-	13.34b	6.15 c
10	avg	-	-	14.91b	7.29 bc
11	avg	-	-	15.57b	8.00 b
avg§	DR	-	-	-	9.2 a
avg	WW	-	-	-	7.0 b

† Different letters indicate different means as determined by a protected LSD (P<0.05).

‡Averaged over water treatment.

§ Averaged over NIL set.

¶Averages for main effects are not presented when interactions were significant, averages for interactions are not presented when they were not significant.

Table 2.4. Ureide content per plant (total_pl, µmol plant^{-1}) of soybean near-isoline sets grown under well watered or water deficit conditions for 10 days prior to harvest at V6 in 2010 growth chamber experiment. Near-isolines were selected for alleles for either high or low ureide concentrations (expected ureide concentration) from a recombinant inbreed population.

Water trt †	NIL set	Expected‡	Total_pl
DR	8	High	129.73 b§
DR	8	Low	156.89 ab
DR	9	High	60.33 ef
DR	9	Low	62.52 ef
DR	10	High	102.13 cd
DR	10	Low	103.85 cd
DR	11	High	72.59 e
DR	11	Low	65.57 ef
WW	8	High	172.67 a
WW	8	Low	126.13 bc
WW	9	High	51.18 ef
WW	9	Low	51.76 ef
WW	10	High	83.65 de
WW	10	Low	107.99 cd
WW	11	High	60.03 ef
WW	11	Low	36.60 f

†Water treatment.

‡ Expected ureide concentration.

§ Different letters indicate different means as determined by a protected LSD (P<0.05).

Discussion

In 2011, recombinant inbreed lines were genotyped again using 494 polymorphic single-nucleotide polymorphism (SNPs) and 170 polymorphic SSRs (Hwang et al., 2013). Quantitative trait loci associated with shoot ureide concentration were detected by composite interval mapping (CIM) and multiple interval mapping (MIM). New QTL analysis detected QTLs more significantly associated with shoot ureide concentration than preliminary QTLs. The new QTLs detected were not located in proximity to Satt 220 or Satt 306. This explains the absence of differences among the NILs. Near-isogenic lines were not included in further experimentation.

CHAPTER III

SELECTING DROUGHT TOLERANT SOYBEAN GENOTYPES USING QTLS ASSOCIATED WITH SHOOT UREIDE AND NITROGEN CONCENTRATIONS

ABSTRACT

In soybean, nitrogen fixation is sensitive to drought, even more so than other physiological processes such as photosynthesis. Drought sensitivity of nitrogen fixation has been associated with high shoot concentrations of ureides and nitrogen. Quantitative trait loci (QTLs) associated with shoot ureide and nitrogen concentration were detected by Hwang et al. (2013) in a KS4895 by Jackson population. The present research evaluated the use of these QTLs in the selection of drought tolerant genotypes. Our objectives were to compare actual versus expected phenotype of recombinant inbreed lines (RILs) selected using molecular markers, and to evaluate the effect of shoot nitrogen and ureide concentrations on nitrogen fixation and yield under well-watered (WW) and drought (DR) conditions. In 2011, a field experiment was conducted in Fayetteville using 12 RILs selected using preliminary QTL information. Selection based on preliminary QTLs did not result in the expected phenotypes for ureide and nitrogen concentrations. Under severe drought conditions, however, genotypes with low WW ureide and nitrogen concentrations had an increase in growth rate, nitrogen fixation rate, and yield (r^2>0.50, P<0.001). In 2012, field experiments were conducted in Fayetteville and Keiser. Recombinant inbreed lines were selected for extreme values of shoot ureide concentration using the QTL information of Hwang et al. (2013). Those RILs selected with alleles for high (or low) ureide and nitrogen concentration had the expected phenotype. Under WW conditions, RILs with alleles for high ureide and nitrogen concentration had higher nitrogen fixation rates, higher percentages of nitrogen derived from the atmosphere, and higher yields than RILs with alleles for low ureide and nitrogen concentrations (r^2>0.20, P<0.0001). Since, under WW conditions nitrogen and yield may be positively correlated, genotypes with low WW ureide and nitrogen and high yield

need to be identified and tested under drought conditions. The QTLs detected by Hwang et al. (2013) can assist on the identification of these genotypes.

Introduction

Drought is the major limitation to crop yields world-wide (Boyer, 1982). In soybean, nitrogen fixation is sensitive to drought, even more so than other physiological processes such as photosynthesis (Kuo and Boersma, 1971; Sinclair, 1986; Durand et al., 1987, Djekoun and Planchon, 1991). In this regard, there are reports of soybean yield increases in response to nitrogen fertilization under water limited conditions but smaller or no yield response was found under well watered conditions (Lyons and Early, 1952; Sorensen and Penas, 1978; Purcell and King, 1996; Purcell et al., 2004; Ray et al., 2006). These results provide evidence that increasing the tolerance of nitrogen fixation to drought will increase yield under water deficit conditions.

The physiological basis of the inhibition of nitrogen fixation under drought is not yet clearly understood. In early research it was hypothesized that under drought, shoot ureide accumulation triggered a feedback mechanism inhibiting nitrogen fixation in the nodules (Purcell et al., 2000). More recent studies showed that shoot ureides were not directly involved in the inhibition of nitrogen fixation under drought (King and Purcell, 2005; Ladrera et al. 2007). Although shoot ureides are not directly involved in the inhibition of nitrogen fixation (King and Purcell, 2005), there is an association among shoot ureide concentration, shoot nitrogen concentration, and the sensitivity of nitrogen fixation to drought (King and Purcell, 2006).

King and Purcell (2006) found that following a 16 to 22 day water deficit period, nitrogen concentration of genotypes with low nitrogen concentration under well watered (WW) conditions was similar to that under well watered conditions. In contrast, nitrogen concentration of genotypes with high nitrogen concentration under well watered conditions decreased in response to drought (DR). This was then confirmed in a greenhouse study, where the only source of nitrogen was through nitrogen fixation, shoot nitrogen concentration under water deficit

conditions increased for six genotypes with low nitrogen concentration under well watered conditions but decreased for two genotypes with high nitrogen concentration under well watered conditions (King and Purcell, 2006).

More recently, King et al. (2013) selected 22 plants introductions with high yield potential and with extreme shoot nitrogen concentrations, grew them in a growth chamber with soil drying conditions and measured nitrogen fixation by the acetylene reduction assay (ARA). They found that the breakpoint in soil water content at which nitrogen fixation was decreased by drought was lower in the genotypes with low nitrogen concentration under well-watered conditions than those genotypes with high nitrogen concentration. A similar relationship was found with drought sensitivity and shoot ureide concentration. Therefore, the sensitivity of nitrogen fixation to drought was positively related to the shoot nitrogen concentration under well-watered conditions and with the shoot ureide concentration under well-watered and drought conditions.

Ray (1987) evaluated the performance of 28 genotypes in response to water stress in a field experiment. Selecting eight of these cultivars, Sall and Sinclair (1991) compared nitrogen accumulation under drought in a field experiment. In that experiment, Jackson (PI 548657, maturity group VIII, Johnson, 1958) was identified as a cultivar with drought tolerant nitrogen fixation. The drought tolerant nitrogen fixation of Jackson was confirmed in greenhouse (Sall and Siclair, 1991; Serraj and Sinclair, 1996; Purcell et al., 1997, 2000) and field experiments (Serraj and Sinclair, 1997). Jackson is an old, low yielding cultivar, with low nitrogen and ureide concentrations under well watered conditions (Purcell et al., 1997). The low nitrogen concentration under well watered conditions may be the basis of its ability to fix nitrogen under drought.

Purcell et al. (1997), in a greenhouse experiment, evaluating nitrogen accumulation under water deficit conditions of six genotypes, identified KS4895 (PI 595081, maturity group IV, Schapaugh and Dille, 1998) as a cultivar with drought sensitive nitrogen fixation. KS4895 is a high-yielding cultivar, with high shoot ureide and nitrogen concentrations under well watered conditions. Subsequently, in a growth chamber experiment, Purcell et al. (1997) compared nitrogen fixation rates, by the acetylene reduction assay, of Jackson and KS4895 under well watered and mild drought conditions. Nitrogen fixation rate under water deficit conditions and at the same soil-moisture content was approximately twice as great for Jackson as for KS4895. However, under well watered conditions nitrogen fixation was similar for the two genotypes.

In 1993, KS4895 (female) was crossed with Jackson (male) (Charlson et al., 2009). Seeds were bulked from F_1 plants, and the resulting F_2 plants were advanced by single seed descent to the F_3 or F_5 generations. The progeny were selected for a narrow range of maturity and were advanced to further generations. Seed from individual F_3 or F_5 plants were bulked to develop 17 F_3- and 80 F_5-derived recombinant-inbred lines (RILs).

The recombinant inbred lines were phenotyped between R4 and R5 for shoot nitrogen and ureide concentrations under well watered conditions in field experiments at Fayetteville, AR in 2005, 2007, and 2011. In addition, at Keiser, Arkansas in 2000, RILs were phenotyped at R2 stage under mild drought conditions. Recombinant inbred lines were genotyped with 664 polymorphic markers (Hwang et al., 2013). Five quantitative trait loci (QTLs) associated with shoot ureide concentration and four QTLs associated with nitrogen concentration were detected by composite interval mapping (CIM) (Table 3.1). Multiple interval mapping (MIM) identified two QTLs associated with shoot ureide concentration and one QTL associated with nitrogen concentration. Quantitative trait loci detected by MIM had similar positions as those detected by

Table 3.1. Quantitative trait loci (QTLs) identified by Hwang et al. (2013) from composite interval mapping (CIM) and multiple interval mapping (MIM) analyses for shoot ureide and nitrogen concentrations in a KS4895 x Jackson population under well-watered (WW) and drought conditions (DR).

	Method	Chromosome number	Position (cM)	Year	Water treatment	QTL effect†
Ureide	CIM	6	2	2005	WW	-2.33
		13	9.5	2007	WW	-5.99
		9	65.8	2011	WW	2.53
		13	79	2011	WW	-2.59
		19	124.3	2011	WW	4.52
		9	1	2000	DR	1.58
		19	122.5	2000	DR	-1.67
	MIM	13	79	-	WW	-3.11
		19	124.2	-	WW	2.34
Nitrogen	CIM	13	11.5	2007	WW	-0.16
		13	40.2	2011	WW	-0.09
		13	79	2011	WW	-0.09
		16	126.5	2011	WW	0.12
		13	49.6	2000	DR	-0.06
		17	72.3	2000	DR	0.05
	MIM	13	79	-	WW	-0.08

Table adapted from Hwang et al. (2013).

† QTL effects were defined as the mean of Jackson alleles minus the means of KS4895 alleles.

composite interval mapping.

Using the QTL information, we selected RILs from the KS4895 x Jackson population with alleles associated with either high or low shoot ureide concentration in order to create contrasting phenotypes. We evaluated, shoot ureide concentration, shoot nitrogen concentration, nitrogen fixation rate, and yield under well watered and drought conditions. We hypothesized that by using molecular marker assisted selection we could select genotypes with alleles that would result in low shoot ureide concentration and low nitrogen concentration under well watered conditions, and that those genotypes would have superior yield under drought because of high nitrogen fixation rates.

To test this hypothesis, we set forth two objectives. They were to: 1) compare genotype versus phenotype of the RILs selected, and 2) evaluate the effects of shoot nitrogen concentration and shoot ureide concentration in nitrogen fixation and yield under well watered and drought conditions.

Materials and Methods

Field experiments were conducted in Fayetteville, AR (36° 05' N, 94° 10' W) in 2011 and 2012, and in Keiser, AR (35° 69' N 90° 08' W) in 2012. Each field experiment was divided in two randomized complete blocks (RCB) with four replications each. In Fayetteville, plots were 4 rows wide, 6.1 m long and with 45.7 cm row spacing. In Keiser, plots were one row wide, 6.1 m long, and with 96.5 cm row spacing. At both locations seeding was at a density of 33 seeds m^{-2}.

For each RCB a water treatment was assigned as either well watered (WW) or drought (DR). The drought treatment was initiated after canopy closure. Before canopy closure in the DR treatment, and in the WW treatment during all season, plots were irrigated as required using an overhead sprinkler in Fayetteville and with a lateral-move irrigation system in Keiser. An irrigation scheduling program (Purcell et al., 2007) was used to determine irrigation timing to the plots at a soil moisture deficit of 30 mm in Fayetteville and 50 mm in Keiser. In 2011, in the DR treatment, irrigation was minimized in order to reduce yield by a target of 50 % of the WW yield. In 2012, a milder drought treatment was applied with the purpose of identifying differences among genotypes. In Fayetteville, dielectric probes (ECH_2O EC-5 soil moisture sensor, Decagon Devices Inc., Pullman, WA) were buried at 15 cm and 30 cm in each block to monitor volumetric water content of the soil.

The soil was a Captina slit loam (fine-silty, siliceous, active, mesic Typic Fragiudults) in Fayetteville and a Sharkey silty clay (very-fine, smectitic, Thermic Chromic Epiaquerts) in Keiser. Soils were fertilized to meet soil test recommendations. In Fayetteville in 2012, heavy rains (42 mm) the day after planting caused the soil surface to crust, and a rotary hoe was used to

break the crust to help seedling emergence. After emergence, at each replication, soil samples of the 0-20 cm depth interval were collected and analyzed for total nitrogen and inorganic nitrogen.

In both years at Fayetteville, rye (*Secale cereale* L.) was planted the previous fall as a winter crop to reduce the amount of inorganic nitrogen in the soil. The following spring, rye was mown at heading and removed from the field. Pre-plant herbicides (Metribuzin, 0.42 kg a.i. ha^{-1}, and S-metolachlor 1.6 kg a.i. ha^{-1}) were incorporated to control weed species. Field experiments were sown on 1 June 2011 and 2 June 2012. Herbicides 'Select 2EC' (Clethodim, 26.4% a.i) and 'Basagran' (Bentazon, 44% a.i) were applied at 593 mL ha^{-1} and 709 mL ha^{-1}, respectively, on 24 June, and 25 August in 2011, and in 17 July in 2012. To control insects, the insecticide 'Karate' (Lambda-cyhalothrin, 22.8% a.i.) was applied on 30 August 2011 and 3 September 3 2012 at a rate of 118 mL ha^{-1}. In 2011, on 3 September, the fungicides 'Folicur' (Tebuconazole, 250 g a.i L^{-1}) and 'Headline' (Pyraclostrobin, 23.6% a.i.) were applied at 500 mL ha^{-1} and 876 mL ha^{-1}, respectively, for controlling late season foliar diseases.

In Keiser, the field was planted with corn (*Zea mays* L.) in 2011 and then left fallow during winter. Field experiments were sown on 7 June 2012. Pre-plant herbicides were Metribuzin (550 g a.i. ha^{-1}), Paraquat (30.1% a.i. at 1419 mL ha^{-1}), and Flumioxazin (51% a.i. at 146 mL ha^{-1}). On 28 July S-metolachlor (83% a.i. at 615 mL ha^{-1}) was sprayed. Herbicides 'Select Max' (Clethodim, 12.6 % a.i.) and 'Flexstar' (Fomesan 22.1 % a.i.) were applied on 17 July 2012 at a dosage of 1168 mL ha^{-1} and 710 mL ha^{-1}, respectively.

The genotypes used in the study consisted of 12 RILs in 2011 and 18 RILs in 2012 in Fayetteville (Table 3.2). In the Keiser experiment, RIL number 11 was not included due to insufficient seed. One group of RILs was selected for alleles for low shoot ureide concentration, and the other group was selected for alleles for high ureide concentration (Tables 3.2). In 2011

the RILs were selected based on preliminary QTL and molecular marker data (see Chapter 2). In 2012, selection was based on phenotypic data and more extensive molecular marker data.

For each QTL, Hwang et al. (2013) calculated the additive effects as the mean nitrogen or ureide concentration of the genotypes with Jackson alleles minus the mean of the genotypes with KS4895 alleles. Therefore, if the additive effect for a particular QTL was negative, alleles from Jackson would be expected to decrease the ureide or nitrogen concentration and vice versa. Assuming there were no epistatic effects, summing the effects of the alleles at each of the QTLs detected, we calculated the cumulative additive effect for each genotype. Separate cumulative additive effects were determined for ureide and nitrogen concentration, under WW and DR conditions, and for composite interval mapping (CIM) and multiple interval mapping (MIM) based upon additive effects described by Hwang et al. (2013). These were: ureide cumulative additive effects detected under well watered conditions by composite interval mapping (Ur_CIM_{ww}), ureide cumulative additive effects detected under drought conditions by composite interval mapping (Ur_CIM_{dr}), ureide cumulative additive effects detected under well watered conditions by multiple interval mapping (Ur_MIM_{ww}), nitrogen cumulative additive effects detected under well watered conditions by composite interval mapping (N_CIM_{ww}), nitrogen cumulative additive effects detected under drought conditions by composite interval mapping (N_CIM_{dr}), and nitrogen cumulative additive effects detected under well watered conditions by multiple interval mapping (N_MIM_{ww}) (Tables 3.2, 3.3, 3.4, 3.5, 3.6, and 3.7). The cumulative additive effect is a numerical value that reflects the allelic composition of a given genotype and the expected phenotype for that genotype. The first objective of this research was to compare shoot ureide concentration and nitrogen concentration with the cumulative additive effects of the genotypes selected.

Table 3.2. Shoot ureide additive effects (µmol g^{-1}) for the five QTLs detected by Hwang et al. (2013) by composite interval mapping under well watered conditions and the cumulative additive effect (Ur_CIM_{ww}) of each of the recombinant inbred lines (RILs) in the study.

		QTLs					
		1	2	3	4	5	Ur_CIM_{ww}
Year	RIL	Additive effect (µmol g^{-1})†					(µmol g^{-1})‡
2011	72	1.16	3	1.3	1.26	2.26	8.98
2011	77	-1.16	3	1.3	1.26	2.26	6.66
2011	71	1.16	3	-1.3	1.26	2.26	6.38
2011	20	-1.16	3	1.3	-1.26	2.26	4.14
2011	76	-1.16	3	1.3	-1.26	2.26	4.14
2011	97	-1.16	3	1.3	-1.26	2.26	4.14
2011-2012	66	-1.16	3	1.3	-1.26	2.26	4.14
2011	59	1.16	-	-1.3	1.26	2.26	3.38
2011	87	1.16	3	1.3	-1.26	-2.26	1.94
2011	80	1.16	-3	1.3	1.26	-2.26	-1.54
2011	10	-1.16	-3	1.3	-1.26	2.26	-1.86
2011	4	-1.16	-3	-1.3	1.26	2.26	-1.94
2012	11	1.16	3	1.3	1.26	2.26	8.98
2012	45	1.16	3	1.3	1.26	2.26	8.98
2012	55	1.16	3	1.3	1.26	2.26	8.98
2012	68	1.16	3	1.3	1.26	2.26	8.98
2012	70	1.16	3	1.3	1.26	2.26	8.98
2012	117	1.16	3	1.3	1.26	2.26	8.98
2012	58	-	3	1.3	1.26	2.26	7.82
2012	33	-	3	1.3	-1.26	2.26	5.3
2012	50	1.16	3	1.3	-1.26	-	4.2
2012	22	-1.16	-3	1.3	-	2.26	-0.6
2012	69	-1.16	-3	-1.3	1.26	2.26	-1.94
2012	17	-1.16	-3	-1.3	-1.26	2.26	-4.46
2012	88	-1.16	-3	-1.3	-1.26	2.26	-4.46
2012	119	-1.16	-3	-1.3	-1.26	2.26	-4.46
2012	83	-1.16	-3	1.3	-1.26	-2.26	-6.38
2012	79	1.16	-3	-1.3	-1.26	-2.26	-6.66
2012	110	-1.16	-3	-1.3	-1.26	-2.26	-8.98

† Additive effects were calculated by dividing each QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

‡ Cumulative additive effect was calculated by summing the additive effects of each QTL.

Table 3.3. Shoot ureide additive effect (µmol g^{-1}) for both QTLs detected by Hwang et al. (2013) by composite interval mapping (CIM) under drought conditions and the cumulative additive effect (Ur_CIM_{dr}) of the recombinant inbreed lines (RILs) in the study.

		QTLs		
		1	2	Ur_CIM_{dr}
Year	RIL	Additive effect (µmol g^{-1}) †		(µmol g^{-1})
2011	87	0.79	0.83	1.62
2011	20	0.79	-	0.79
2011	80	-0.79	0.83	0.04
2011	4	0.79	-0.83	-0.04
2011	71	0.79	-0.83	-0.04
2011	72	0.79	-0.83	-0.04
2011	77	0.79	-0.83	-0.04
2011	10	-0.79	-0.83	-1.62
2011	59	-0.79	-0.83	-1.62
2011	76	-0.79	-0.83	-1.62
2011	97	-0.79	-0.83	-1.62
2011-2012	66	-0.79	-0.83	-1.62
2012	45	0.79	0.83	1.62
2012	79	0.79	0.83	1.62
2012	69	-0.79	0.83	0.04
2012	83	-0.79	0.83	0.04
2012	110	-0.79	0.83	0.04
2012	117	0.79	-0.83	-0.04
2012	119	0.79	-0.83	-0.04
2012	50	-	-0.83	-0.83
2012	58	-	-0.83	-0.83
2012	11	-0.79	-0.83	-1.62
2012	22	-0.79	-0.83	-1.62
2012	33	-0.79	-0.83	-1.62
2012	55	-0.79	-0.83	-1.62
2012	68	-0.79	-0.83	-1.62
2012	70	-0.79	-0.83	-1.62
2012	88	-0.79	-0.83	-1.62

†Additive effects were calculated by dividing each QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

‡ Cumulative additive effect was calculated by summing the additive effects at each QTL.

Table 3.4. Shoot ureide additive effect (µmol g^{-1}) for both QTLs detected by Hwang et al. (2013) by multiple interval mapping (MIM) and the cumulative additive effect (Ur_MIM_{ww}) of the recombinant inbred lines (RILs) in the study.

		QTLs		
		1	2	Ur_MIM_{ww}
Year	RIL	Additive effect (µmol g^{-1})†		(µmol g^{-1})‡
2011	72	1.3	1.17	2.47
2011	20	1.3	1.17	2.47
2011	76	1.3	1.17	2.47
2011	97	1.3	1.17	2.47
2011	87	1.3	1.17	2.47
2011	80	1.3	-1.17	0.13
2011	77	-1.3	1.17	-0.13
2011	71	-1.3	1.17	-0.13
2011-2012	66	-1.3	1.17	-0.13
2011	59	-1.3	1.17	-0.13
2011	4	-1.3	1.17	-0.13
2011	10	-1.3	-1.17	-2.47
2012	11	1.3	1.17	2.47
2012	45	1.3	1.17	2.47
2012	55	1.3	1.17	2.47
2012	68	1.3	1.17	2.47
2012	70	1.3	1.17	2.47
2012	117	1.3	1.17	2.47
2012	58	1.3	1.17	2.47
2012	69	1.3	1.17	2.47
2012	22	-	1.17	1.17
2012	33	-1.3	1.17	-0.13
2012	17	-1.3	1.17	-0.13
2012	88	-1.3	1.17	-0.13
2012	119	-1.3	1.17	-0.13
2012	50	-1.3	-	-1.3
2012	83	-1.3	-1.17	-2.47
2012	79	-1.3	-1.17	-2.47
2012	110	-1.3	-1.17	-2.47

†Additive effects were calculated by dividing each QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

‡Cumulative additive effect was calculated by summing the additive effects of each QTL.

Table 3.5. Shoot nitrogen additive effects (% N) for the four QTLs detected by Hwang et al. (2013) by composite interval mapping (CIM) under well watered conditions and the cumulative additive effect (N_CIM_{ww}) of the recombinant inbred lines (RILs) in the study.

		QTLs				
		1	2	3	4	N_CIM_{ww}
Year	RIL	Additive effect (% N) †				(% N)‡
2011	20	0.08	0.045	0.045	-0.06	0.11
2011	77	0.08	0.045	0.045	-0.06	0.11
2011	87	0.08	0.045	0.045	-0.06	0.11
2011	97	0.08	0.045	0.045	-0.06	0.11
2011	71	0.08	0.045	-0.05	-0.06	0.02
2011	72	0.08	-0.045	0.045	-0.06	0.02
2011	76	0.08	-0.045	0.045	-0.06	0.02
2011-2012	66	0.08	-0.045	0.045	-0.06	0.02
2011	59	-	0.045	-0.05	-	0
2011	80	-0.08	0.045	0.045	-0.06	-0.04
2011	10	-0.08	-0.045	0.045	-0.06	-0.14
2011	4	-0.08	-0.045	-0.05	-0.06	-0.23
2012	11	0.08	0.045	0.045	0.06	0.23
2012	45	0.08	0.045	0.045	0.06	0.23
2012	58	0.08	0.045	0.045	0.06	0.23
2012	55	0.08	-	0.045	0.06	0.19
2012	68	0.08	0.045	0.045	-0.06	0.11
2012	70	0.08	0.045	0.045	-0.06	0.11
2012	33	0.08	-0.045	0.045	-	0.08
2012	117	0.08	-0.045	0.045	-0.06	0.02
2012	50	-	-0.045	0.045	-	0
2012	69	-0.08	0.045	-0.05	0.06	-0.02
2012	110	-0.08	0.045	-0.05	0.06	-0.02
2012	22	-0.08	-0.045	0.045	0.06	-0.02
2012	119	-0.08	-0.045	-0.05	0.06	-0.11
2012	17	-0.08	-	-0.05	-	-0.13
2012	88	-0.08	0.045	-0.05	-0.06	-0.14
2012	83	-0.08	-0.045	0.045	-0.06	-0.14
2012	79	-0.08	-0.045	-0.05	-0.06	-0.23

† Additive effects were calculated by dividing each QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

‡ Cumulative additive effect was calculated by summing the additive effects at each QTL.

Table 3.6. Shoot nitrogen additive effects (% N) for both QTLs detected by Hwang et al. (2013) by composite interval mapping (CIM) under drought conditions and the cumulative additive effect (N_CIM_{dr}) of the recombinant inbreed lines (RILs) in the study.

		QTLs		
		1	2	N_CIM_{dr}
Year	RIL	Additive effect (%N)†		‡ (% N)
2011	87	-	0.025	0.025
2011	20	0.03	-0.025	0.005
2011	72	0.03	-0.025	0.005
2011	77	0.03	-0.025	0.005
2011	80	0.03	-0.025	0.005
2011	97	0.03	-0.025	0.005
2011	4	-0.03	0.025	-0.005
2011	76	-0.03	0.025	-0.005
2011-2012	66	-0.03	0.025	-0.005
2011	59	-0.03	-	-0.03
2011	10	-0.03	-0.025	-0.055
2011	71	-0.03	-0.025	-0.055
2012	22	0.03	0.025	0.055
2012	55	0.03	0.025	0.055
2012	68	0.03	0.025	0.055
2012	58	0.03	-	0.03
2012	11	0.03	-0.025	0.005
2012	45	0.03	-0.025	0.005
2012	70	0.03	-0.025	0.005
2012	88	0.03	-0.025	0.005
2012	110	0.03	-0.025	0.005
2012	69	-0.03	0.025	-0.005
2012	83	-0.03	0.025	-0.005
2012	117	-0.03	0.025	-0.005
2012	119	-0.03	0.025	-0.005
2012	33	-	-0.025	-0.025
2012	50	-0.03	-0.025	-0.055
2012	79	-0.03	-0.025	-0.055

†Additive effects were calculated by dividing each QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

‡ Cumulative additive effect was calculated by summing the additive effects at each QTL.

Table 3.7. Shoot nitrogen additive effect (N_MIM_{ww}) for the QTL detected by Hwang et al. (2013) by multiple interval mapping (MIM) of the recombinant inbred lines (RILs) in the study.

Year	RIL	N_MIM_{ww} (% N)
2011	10	0.04
2011	80	0.04
2011	72	0.04
2011	76	0.04
2011	20	0.04
2011	77	0.04
2011	87	0.04
2011	97	0.04
2011-2012	66	0.04
2011	4	-0.04
2011	59	-0.04
2011	71	-0.04
2012	83	0.04
2012	22	0.04
2012	50	0.04
2012	117	0.04
2012	33	0.04
2012	68	0.04
2012	70	0.04
2012	55	0.04
2012	11	0.04
2012	45	0.04
2012	58	0.04
2012	79	-0.04
2012	88	-0.04
2012	17	-0.04
2012	119	-0.04
2012	69	-0.04
2012	110	-0.04

† Additive effects were calculated by dividing the QTL effect by two and assigning a positive sign if the allele increased ureide concentration or negative sign if the allele decreased ureide concentration.

Samples for shoot nitrogen concentration and shoot ureide concentration were collected during the season. Three plants from each plot were cut at ground level, dried in an oven at 65°C for one week, coarse ground through a 6 mm screen and then fine ground through a 0.425 mm screen. Subsamples of 125 mg were used for ureide extraction, and quantification was done using the colorimetric procedure of Young and Conway (1942) as described by de Silva et al. (1996). A second set of subsamples of 125 mg was used to determine nitrogen concentration using the Dumas combustion method by the Soil Test and Plant Analysis Laboratory at the University of Arkansas.

In Fayetteville nitrogen fixation rates were determined using the N difference method as described by Peoples et al. (2009). In the two middle rows of each plot, a 1 m^2 biomass sample was collected by cutting the plants at the ground level. Samples were dried in an oven at 65°C for 2 weeks and weighed. After recording dry weight (g m^{-2}), nitrogen concentration (%N) and ureide concentration (μmol g^{-1}) were determined by the procedures described previously. Nitrogen content in each sample (mg N m^{-2}) was calculated by multiplying %N by dry weight (g m^{-2}). Mean nitrogen accumulation rate (mg N m^{-2} day^{-1}) was calculated as the difference in nitrogen content (mg N m^{-2}) between sample dates divided by the number of days between sample dates. In 2011 the first biomass sample was collected on 18 July (V8) and the second sample on 8 August (R2), while in 2012 the first biomass sample was collected on 7 August (R2) and the second on 5 September (R5). In 2011, a rainfall event (85 mm in 6 days) was predicted, and we sampled early on 7 August (R2) in order to have a water deficit effect in the drought treatment. In 2012, we collected the second biomass sample on 5 September (R5) stage, the period when most of the phenotypic data for the QTL analysis were collected.

A non-nodulating genotype (PI 573285, Hartwig, 1994) was planted in each replication both years at Fayetteville and Keiser. In Fayetteville, the non-nodulating genotype was used for estimating the N uptake from the soil (mg N m^{-2}). By subtracting the estimated nitrogen content of the non-nodulating genotype from the nitrogen content of the RILs the nitrogen fixation rate (mg N m^{-2} day^{-1}) for each plot was estimated.

At Keiser, the fraction of nitrogen derived from the atmosphere (%Ndfa) was measured using the ^{15}N natural abundance method as described by Peoples et al. (2009). This method is based on the different ^{15}N:^{14}N ratio between the atmospheric nitrogen and the plant-available soil nitrogen. The relative deviation (in ‰) from the ratio ^{15}N:^{14}N in atmospheric nitrogen is expressed as $\delta^{15}N$. Estimates of %Ndfa were calculated as:

$$\%\text{Ndfa} = \frac{100\ (\delta^{15}\text{N of the non nodulating genotype} - \delta^{15}\text{N of the sample})}{\delta^{15}\text{N of the non nodulating genotype} - \delta^{15}\text{N of soybean without soil N}} \quad (1)$$

Oberson et al. (2007) found that $\delta^{15}N$ of soybean leaf tissue from inoculated plants growing in a media without mineral nitrogen was -2, and we used this value for estimating %Ndfa. Samples consisted of three central leaflets collected from the most developed leaf from three individual plants. After collection, samples were dried and ground through a 0.425 mm screen. Subsamples of 3 to 8 mg were sent to the University of California (UC Davis) Stable Isotope Facility for isotopic analysis by a continuous flow Isotope Ratio Mass Spectrometer (http://stableisotopefacility.ucdavis.edu/).

In Fayetteville at harvest maturity, grain was harvested from 2 m^2 of the two middle rows of each plot. Samples were weighed (g m^{-2}) and water content (g g^{-1}) of each sample was determined using a portable grain moisture meter (Multi-Grain portable moisture tester, Dickey-john® Co., Auburn, IL). Grain yield (g m^{-2}) was standardized to 13 % moisture.

Statistical analysis was done using the SAS 9.2 (2008) statistical software package (SAS Insititute Inc., Cary, NC, USA). A general linear model was used for analysis of variance. Means were separated by Fisher's protected least significance difference (LSD, P=0.05).

Analysis of covariance was used to evaluate various responses to the different measures of cumulative additive effects using water treatment or developmental stage as covariates. Linear, quadratic models and interactions were tested. Non significant higher order effects and interactions were eliminated and the model was reevaluated. The genotype effect was analyzed using the cumulative additive effects of each genotype that was associated with ureide or nitrogen concentrations. Simple linear and quadratic regressions were determined, using the cumulative additive effects as independent variables and shoot ureide and nitrogen concentration as dependent variables.

Results

Fayetteville 2011

In the Fayetteville 2011 growing season average temperature was 24.5°C with a peak of mean daily temperature of 35°C around R2 (Figure 3.1). These high temperatures at flowering may have had an effect on pod development since the period between full flowering (R2) and full pod (R4) was longer than expected (Van Schaik and Probst, 1958; Mann and Jaworski, 1970). Temperatures decreased markedly starting at R4.

The volumetric water content averaged across all sample dates and depths was 0.14 cm^3 cm^{-3} and 0.24 cm^3 cm^{-3} for the drought (DR) and the well watered (WW) treatments, respectively. A large rainfall (85 mm) at 67, 68 and 69 days after emergence (DAE) increased the volumetric water content for both water treatments (Figure 3.2). The second biomass harvest (H_2) was done prior to the rainfall event, when the soil in the DR treatment was still dry.

Shoot ureide and nitrogen concentration

Shoot ureide and nitrogen concentrations were affected by water treatment, developmental stage, and genotype, and the interaction among these three factors was significant (Appendix table A). Since the three-way interaction was significant, we investigated at each developmental stage, the effects and interactions of the water and genotype treatments (Appendix table B). There were significant interactions between water treatment and genotype for shoot nitrogen concentration at sample dates 23 DAE (V5) and 36 DAE (V7), and for shoot ureide concentration at all sample dates except at 51 DAE (R2) and 64 DAE (R3). When the interaction was not significant, means were separated and evaluated for the main effects as appropriate.

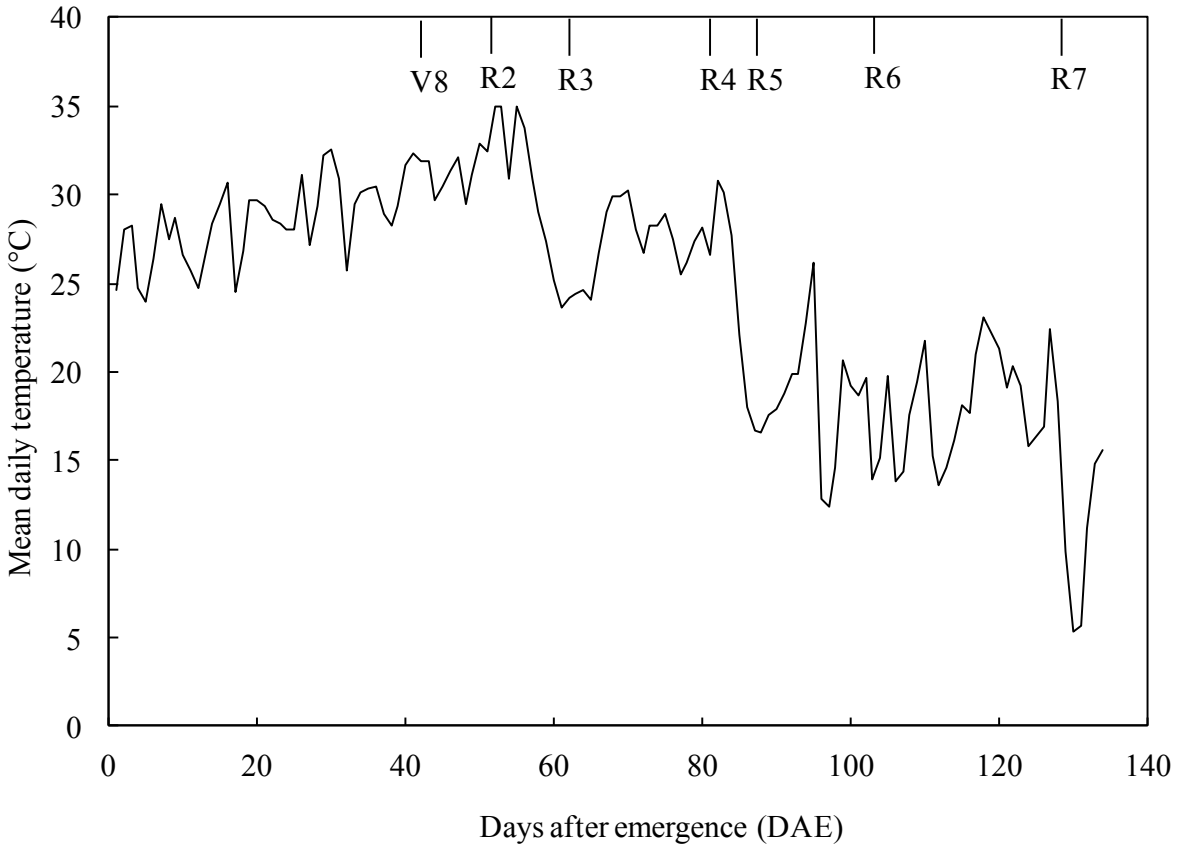

Figure 3.1. Mean daily temperature of Fayetteville 2011 versus days after emergence. Emergence was on 5 June 2011.

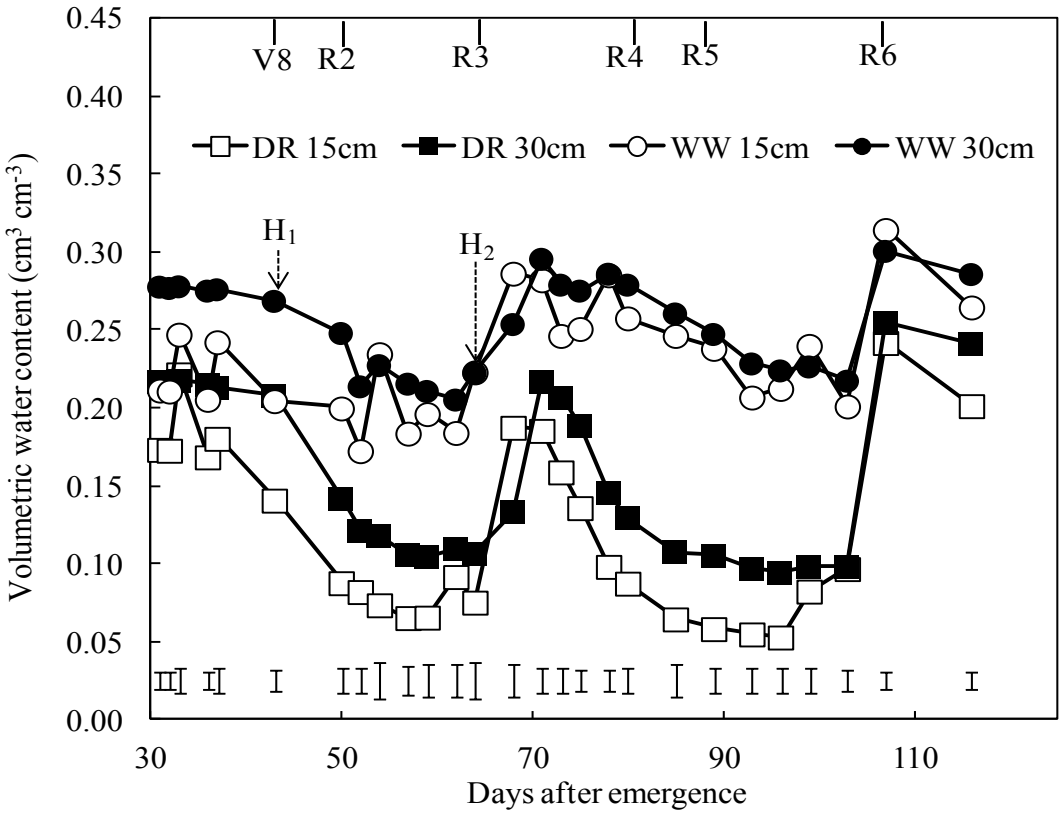

Figure 3.2. Volumetric water content ($cm^3\ cm^{-3}$) at two depths (15 cm and 30 cm) in the well-watered (WW) and drought (DR) treatments in Fayetteville 2011. Each data point is the mean of 4 replications and bars represent the standard errors averaged across depths and water treatments. Biomass harvest 1 (H_1), harvest 2 (H_2), and significant phenological stages are indicated near the top of the figure.

Water treatment significantly affected both variables for all samples dates except for shoot ureide concentration at 36 DAE (V7), 51 DAE (R2), and 64 DAE (R3), and shoot nitrogen concentration at 23 DAE (V5), 51 DAE (R2) and 82 DAE (R4) (Appendix table B). Since water treatment had two levels (WW and DR), significant differences between water treatments were determined by an F-test. Shoot nitrogen concentration was higher in the DR treatment than in the WW treatment at 43 DAE (V8) and at 64 DAE (R3) (Figure 3.3); at 51 DAE (R2) and 82 DAE (R4) there were no significant differences between water treatments; and at 89 (early R5), 96 (late R5), and 108 DAE (R6) shoot nitrogen concentration was higher in the WW treatment than the DR treatment. In both water treatments shoot nitrogen concentration decreased rapidly from 43 DAE (V8) to 51 DAE (R2) and then continued decreasing but at a slower rate to the end of the measurement period (108 DAE).

A summary of the sources of variation for the responses of shoot ureide and nitrogen concentration versus the various cumulative additive effects are given in the appendix (Appendix tables C to H). In Fayetteville 2011, there were no significant relationships between ureide or nitrogen concentration and the additive effects for most of the sample dates. For the significant relationships, where there was no interaction between the additive effect and water treatment but main effects were significant, water treatment influenced only the intercept of the equations and consequently the slopes were identical between the WW and DR treatment (Table 3.8).

Nitrogen concentration at various developmental stages had significant relationships with N_CIM_{ww}, N_CIM_{dr}, and N_MIM_{ww} with R^2 values ranging from 0.08 to 0.34 (Table 3.8). Linear regressions had the same slope under well-watered and drought conditions, showing that shoot nitrogen concentration response to the additive effects was similar under both water treatments.

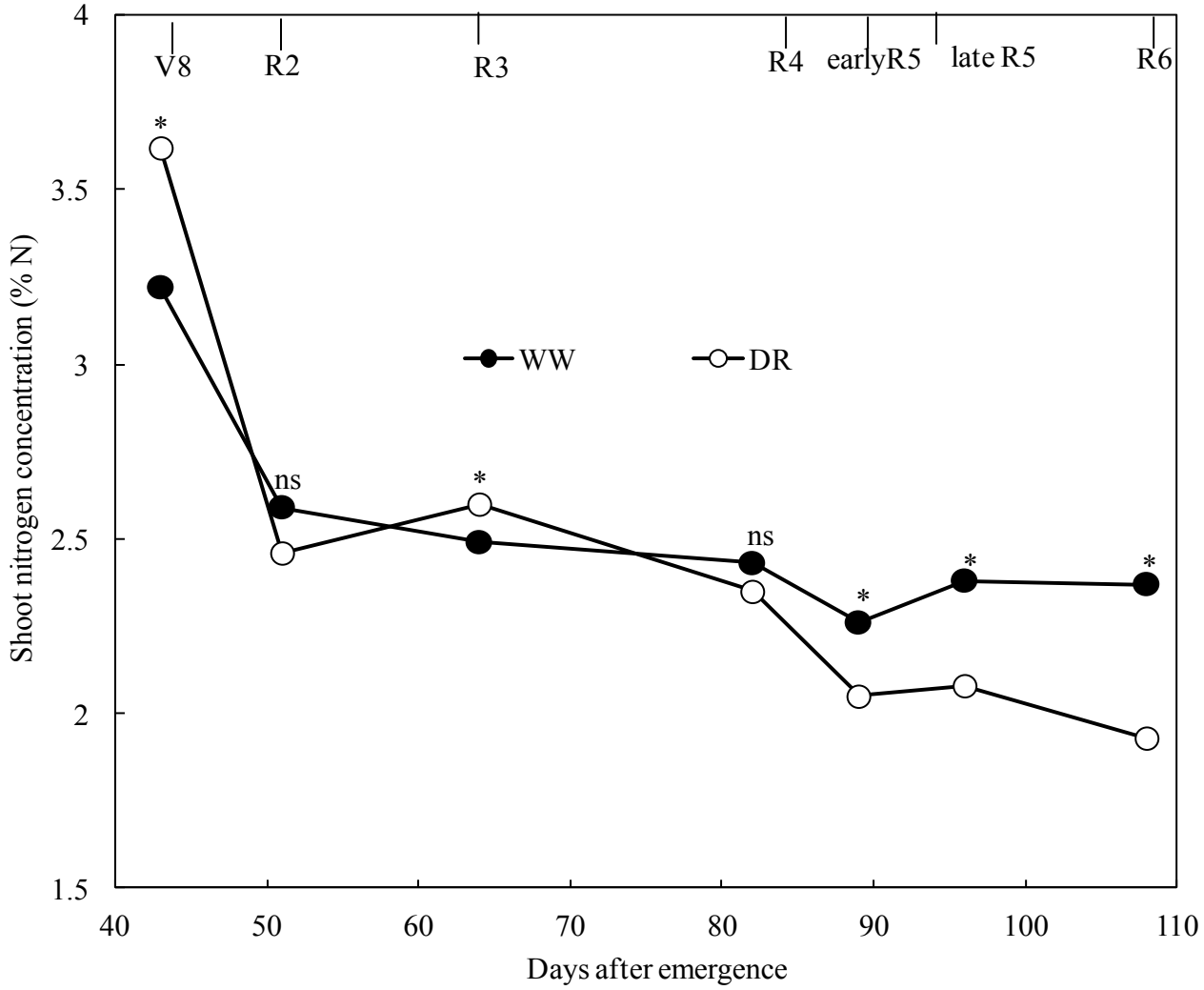

Figure 3.3. Shoot nitrogen concentration in the drought (DR) and well-watered (WW) treatments, averaged over genotypes, versus days after emergence in Fayetteville 2011. Significant differences (*) were detected using an F-test (P=0.05) and displayed in the figure along with non significant (ns) differences.

Table 3.8. Shoot nitrogen concentration in Fayetteville 2011 versus nitrogen cumulative additive effects detected: under well watered (WW) conditions by composite interval mapping (N_CIM_{ww}), under drought (DR) conditions by composite interval mapping (N_CIM_{dr}), and under well watered conditions by multiple interval mapping (N_MIM_{ww}). Water treatment as a covariate was included when significant, otherwise water treatments were analyzed together using linear regression.

Additive effect	Dev. stage	Water trt.	Parameter estimates†				Model significance
			β_0	β_1	β_2	R^2	
N_CIM_{ww}	V5	both‡	4.10***	0.66**		0.08	<0.001
N_CIM_{dr}	V7	DR	3.21***	9.81**	219**	0.28	<0.01
N_MIM_{ww}	V5	both	4.05***	2.39***		0.16	<0.0001
	V7	WW	3.20***	2.81**		0.27	<0.0001
		DR	3.45***	2.81**		0.27	<0.0001
	R2	WW	2.56***	1.33*		0.11	<0.01
		DR	2.43***	1.33*		0.11	<0.01
	R3	both	2.51***	1.71**		0.10	<0.001
	late R5	WW	2.36***	1.14*		0.34	<0.0001
		DR	2.05***	1.14*		0.34	<0.0001

† Quadratic responses ($y= \beta_0+\beta_1 x+\beta_2 x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

‡ Values of both water treatments are presented when the water treatment effect was not significant.

Ns= no significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

There were significant relationships with poor fit ($R^2 \leq 0.24$) between the shoot ureide concentration at various developmental stages and Ur_CIM_{ww} and Ur_CIM_{dr} (Table 3.9). The regression with the highest R^2 and the lowest *P*-value was shoot ureide concentration under drought versus Ur_CIM_{dr} at the R2 developmental stage. The better fit at R2 may be because at R2 was when phenotypic data for drought QTLs were originally collected. The regression had a positive slope showing that as Ur_CIM_{dr} increased, ureide concentration under drought increased as well (Figure 3.4). Although, there were linear models with high r^2 using Ur_MIM_{ww}, most of the regressions did not have significant slopes and the intercept was the only significant term (Tables 3.9).

In 2011, selection was based upon preliminary QTL data, and RILs did not have as extreme values of additive effects as the RILs evaluated in 2012. The narrow range of additive effects of the genotypes likely contributed to the absence of significant coefficients and low r^2 values in the regressions.

Nitrogen Fixation and Yield

In Fayetteville 2011 nitrogen fixation rate (mg N m^{-2} day^{-1}) and grain yield (g m^{-2}) were greater in the well watered treatment than in the drought treatment (Figures 3.5), and there was no significant difference among genotypes or interaction between genotype and water treatment (Appendix tables I and J).

Nitrogen fixation rate (mg N m^{-2} day^{-1}) was negative in the drought treatment (Figure 3.5 A). This negative nitrogen fixation rate was attributable to a senescence of leaf and petiole material under intense drought conditions during the period starting at the first biomass harvest (H_1) and ending at the second biomass harvest (H_2) (Figure 3.2). Negative biomass accumulation rates resulted in negative nitrogen fixation rates during the period. In the drought treatment,

Table 3.9. Shoot ureide concentration in Fayetteville 2011 versus ureide cumulative additive effects detected: under well watered (WW) conditions by composite interval mapping (Ur_CIM_{ww}), under drought conditions (DR) by composite interval mapping (Ur_CIM_{dr}), and under well watered conditions by multiple interval mapping (Ur_MIM_{ww}). Water treatment as a covariate was included when was significant, otherwise water treatments were analyzed together using linear regression.

Additive effect	Dev. stage	Water trt	Parameter estimates†			R^2	Model significance
			β_0	β_1	β_2		
Ur_CIM_{ww}	V7	both‡	16.0***	-0.80***	0.09**	0.19	<0.001
Ur_CIM_{dr}	V5	DR	12.7***	0.82*		0.14	<0.05
	V8	DR	24.8***	2.11**		0.21	<0.01
	R2	DR	17.2***	1.22**		0.24	<0.001
	R3	DR	14.7***	0.15^{ns}	1.23**	0.24	<0.01
	R4	DR	20.24***	2.23**		0.19	<0.001
Ur_MIM_{ww}	V7	both	15.9***	-0.56*		0.07	<0.05
	early R5	WW	45.1***	-0.77^{ns}		0.77	<0.0001
		DR	20.5***	1.74*		0.77	<0.0001
	late R5	WW	51.2***	-1.09^{ns}		0.77	<0.0001
		DR	22.2***	1.11^{ns}		0.77	<0.0001
	R6	WW	33.1***	-1.00^{ns}		0.64	<0.0001
		DR	16.1***	0.69^{ns}		0.64	<0.0001

† Quadratic responses ($y= \beta_0+\beta_1x+\beta_2x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

‡ Values of both water treatments are presented when the water treatment effect was not significant.

Ns= no significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

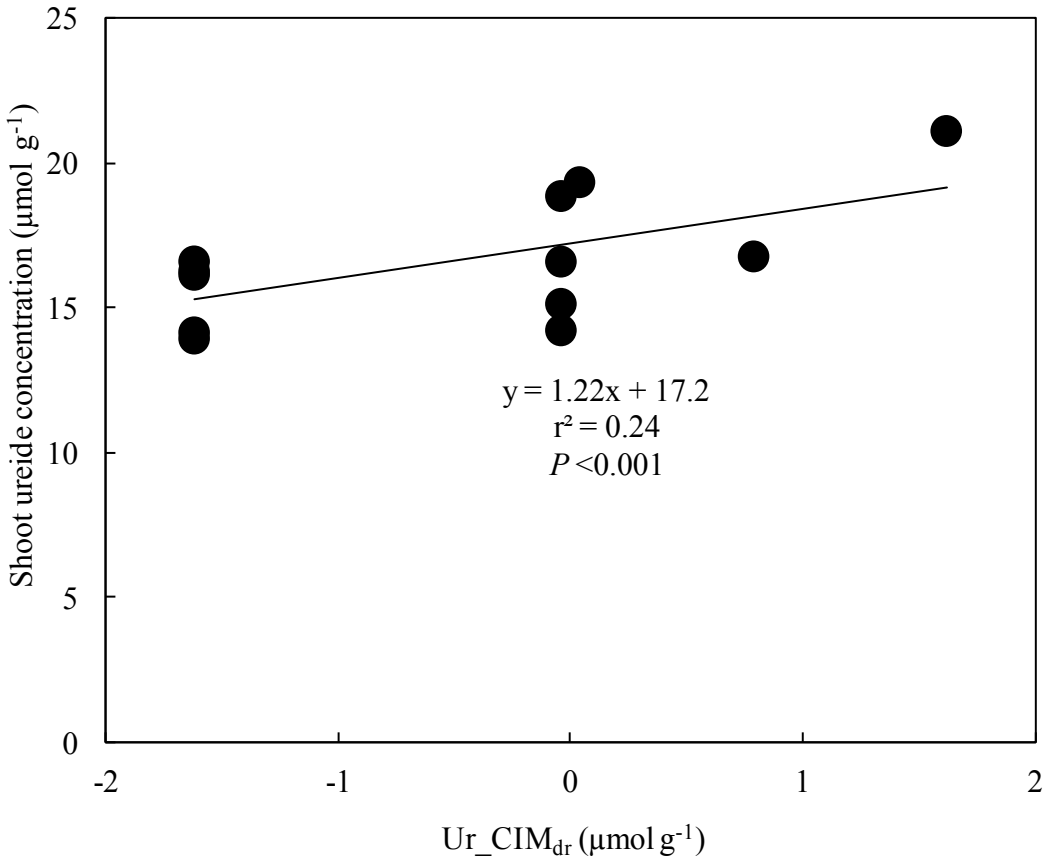

Figure 3.4. Shoot ureide concentration in the drought treatment at R2 stage in Fayetteville 2011 versus ureide cumulative additive effects detected by composite interval mapping under drought conditions (Ur_CIM_{dr}). Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-value, and r^2 were calculated using raw data.

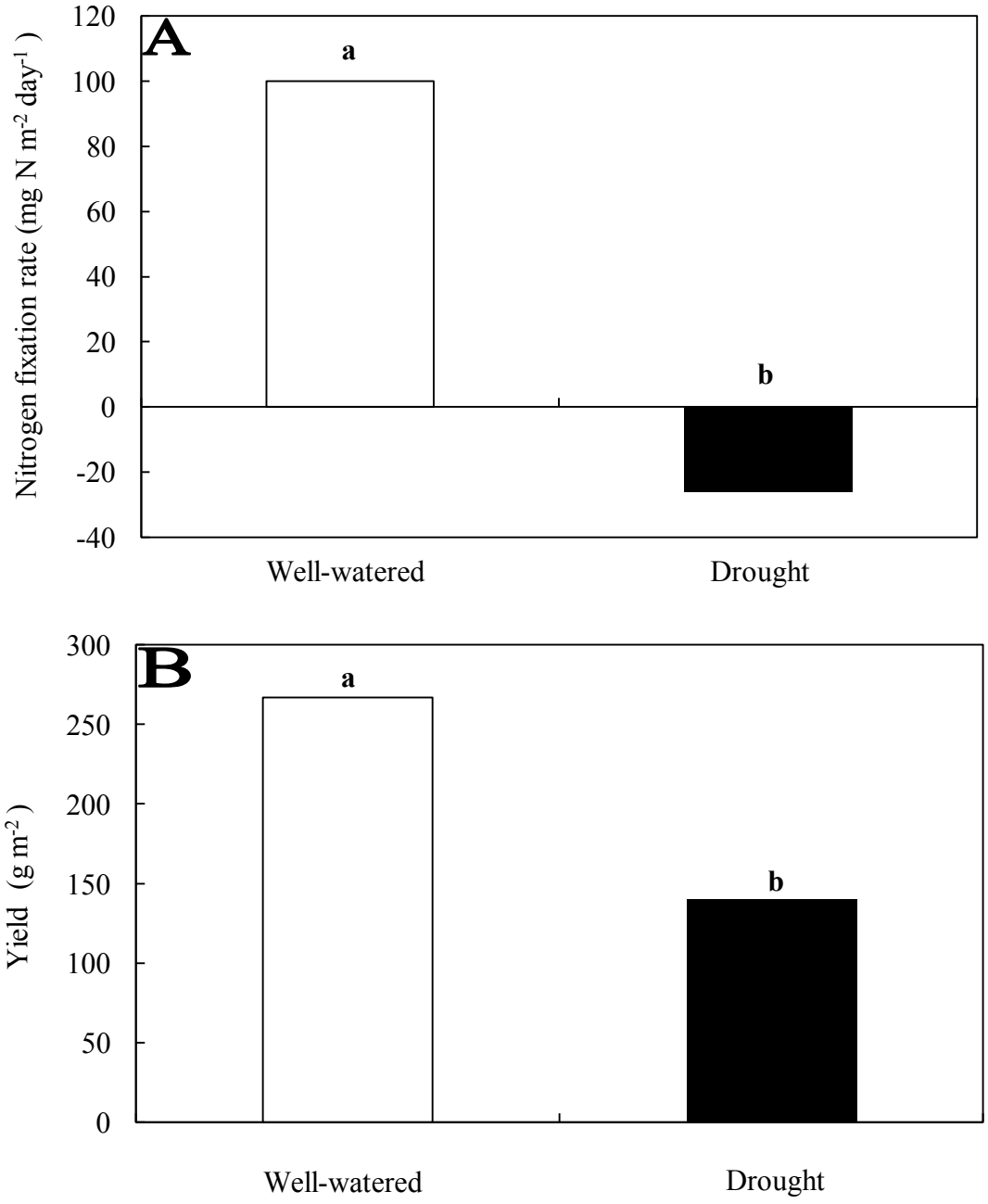

Figure 3.5. A) Nitrogen fixation rate (mg N m^{-2} day^{-1}) from V8-R3 and B) Yield (g m^{-2}) in the well watered and drought treatments averaged over genotypes (genotype x water treatment, ns) for Fayetteville 2011. Different letters indicate different means as determined by a protected LSD (P<0.05).

nitrogen fixation accounted for 73.4% of the nitrogen at the first biomass sample (V8) and 75.5% at the second biomass sample (R3). The DR stress limited nitrogen fixation during this period.

In the well-watered treatment nitrogen fixation rate during the V8-R3 period was 100 mg N m^{-2} day^{-1}. This is low compared with nitrogen fixation rates reported in the literature (Mastrodomenico and Purcell, 2012). Nitrogen fixation increases drastically from R1 reaching its peak at R5 developmental stage (Zapata et al., 1987). In 2011 we measured nitrogen fixation from V8 to R3, when nitrogen fixation was still low. However, since soil nitrogen was depleted by the previous rye crop, at V8 nitrogen fixation accounted for 70.6% of the total shoot nitrogen, and at R3 for 79.3%. We attribute our lower nitrogen fixation rates compared to reports in the literature to differences in developmental stages.

Yield (g m^{-2}) in the well watered treatment averaged 267 g m^{-2} while in the drought treatment averaged 140 g m^{-2} (Figure 3.5 B). This represents a 48% yield decrease as a result of severe drought stress. This was close to our target of 50% of reduction in yield.

Although nitrogen fixation rate and yield did not differ significantly among genotypes, we evaluated possible relationships between nitrogen fixation rate and yield under drought conditions, and: 1) the difference in ureide concentration (ΔU) between the DR and WW treatment, 2) the ureide concentration in the WW treatment (WW U), 3) difference in nitrogen concentration (ΔN) between the DR and WW treatment, and 4) nitrogen concentration in the WW treatment (WW N). Nitrogen fixation rate under drought increased as ΔU increased at V8 and R3 (Table 3.10). The first phenological stage was especially important because at this stage ureide concentration in the DR treatment increased drastically after the initiation of the DR treatment. At this stage, as ureide concentration increased in the DR treatment or decreased in the WW treatment, nitrogen fixation rate under drought conditions increased. This regression

Table 3.10. Simple linear regression data and sample size for the relationships between nitrogen fixation rate (mg N m^{-2} day^{-1}) and yield (g m^{-2}) under drought conditions in Fayetteville 2011, versus well watered ureide and nitrogen concentrations (WW U and WW N), and the difference in ureide and nitrogen concentration between drought conditions and well watered conditions (ΔU and ΔN). Non significant relationships are not reported.

	Nfix vs ΔU	Yield vs WW U	Yield vs WW N	Yield vs ΔU	Yield vs ΔN
Stage	V8	R4	R2	V5	R2
Slope	5.05	-1.32	62.8	7.03	-46.1
Intercept	53	175	-19.3	-136	-137
r^2	0.49*	0.39*	0.56**	0.70**	0.58**
n	11	11	11	11	11
Stage	R3	early R5	R4	late R5	R4
Slope	5.28	-1.24	-69.1	0.83	84
Intercept	24	199	308	-166	-147
r^2	0.37*	0.37*	0.41*	0.42*	0.47*
n	12	11	12	11	12
Stage	average	late R5	late R5		late R5
Slope	5.66	-1.11	-47.8		59.1
Intercept	7.7	199	257		-161
r^2	0.51**	0.71**	0.47*		0.39*
n	12	11	11		11
Stage		average	R6		
Slope		-4.18	-60.7		
Intercept		247	287		
r^2		0.60**	0.82**		
n		11	11		

* and ** indicate regression significant at the 0.05 and 0.01 probability levels.

was highly influenced by RIL number 87 which had the highest ΔU and positive nitrogen fixation rate. When we analyze if high ΔU in RIL 87 was the consequence of a low ureide concentration under well watered conditions (low WW U) or high ureide concentration under drought conditions (high DR U) we found that WW U for RIL 87 was similar to other genotypes (WW U of RIL 87=19.6 μmol g^{-1}, mean of all genotypes=18.4 μmol g^{-1}, SD=2.7 μmol g^{-1}) but DR U was higher for RIL 87 than the other genotypes (DR U of RIL 87=30.9 μmol g^{-1}, mean of all genotypes=22.9 μmol g^{-1}, SD=3.9 μmol g^{-1}). This contrasts with previous reports that showed that under drought the accumulation of ureides inhibited nitrogen fixation (Vadez and Sinclair, 2001; King and Purcell, 2005). Average ΔU over all sample dates showed the strongest relationship with nitrogen fixation under drought (Table 3.10), and there was no relationship with nitrogen fixation under well watered conditions (Figure 3.6). These data show that over the season, genotypes with either low WW U or high DR U, or both, had the highest nitrogen fixation rates under drought.

Yield under drought increased as ureide concentration in the well watered treatment at R4, early R5, and late R5 decreased (Table 3.10.) Also, ureide concentration in the well-watered treatment (WW U) averaged over all sample dates was negatively related with yield in the DR treatment (Figure 3.7 A). Yield under drought conditions was also negatively related to nitrogen concentration in the well-watered treatment (WW N) at R4, late R5, and R6 stages (Table 3.10). At the R2 stage, the relationship between yield in the DR treatment and WW N was positive. The linear regression with the best fit ($p<0.01$ and $r^2=0.82$) was with WW N at R6 (Figure 3.7B).

The difference in ureide concentration between the drought and well-watered treatment (ΔU) at V5 and at late R5 was positively related to yield under drought (Table 3.10). Coefficients of determination (r^2) for these regressions were 0.70 and 0.42 for ΔU at V5 and late R5,

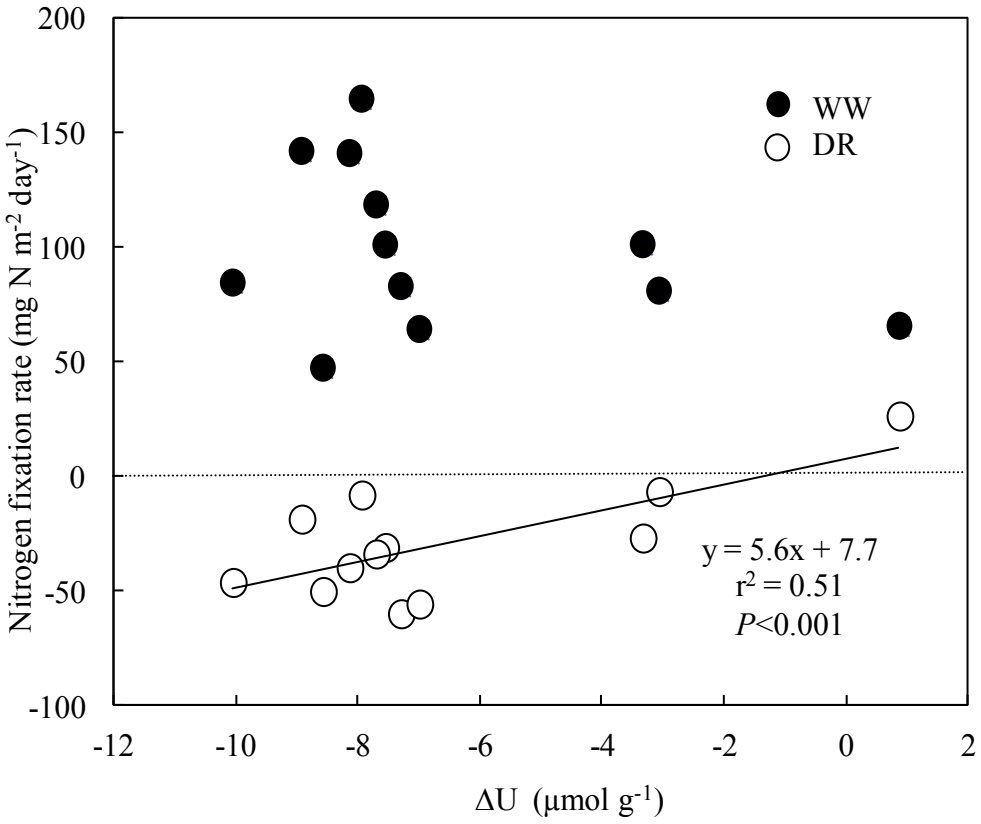

Figure 3.6. Nitrogen fixation rate in the V8-R3 period versus difference in ureide concentration between the drought and well-watered treatment (ΔU) averaged over all sample dates for Fayetteville 2011. Each data point represents one genotype and is the average over 4 replications.

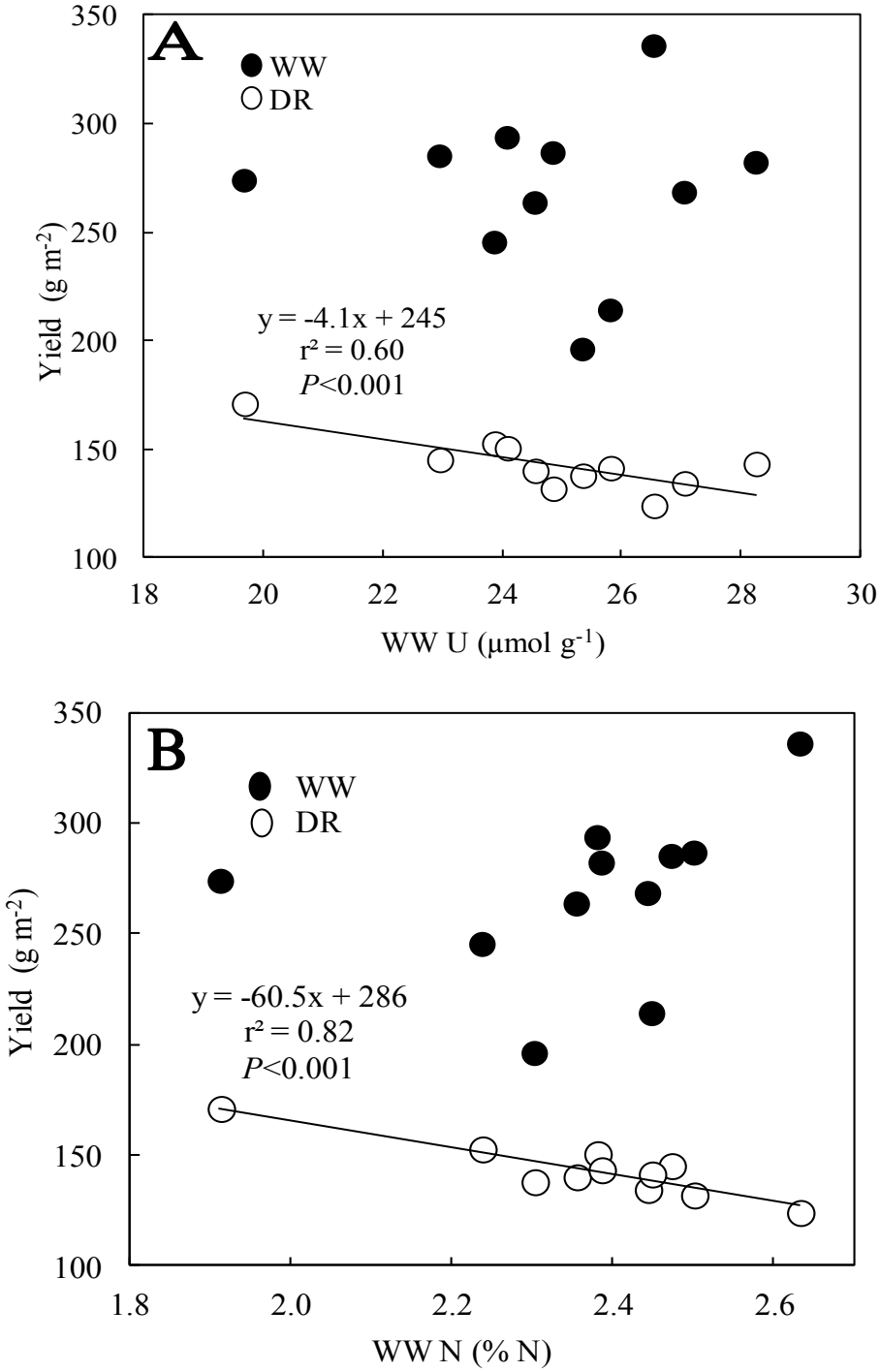

Figure 3.7. Yield (g m^{-2}) in Fayetteville 2011 versus: A) shoot ureide concentration under well-watered conditions (WW U) averaged over all sample dates, and B) shoot nitrogen concentration under well-watered (WW N) conditions at R6 stage. Each data point represents one genotype and is the average over 4 replications.

respectively. As ureide concentration decreased in the well-watered treatment, or increased in the drought treatment, or both, yield in the DR treatment increased.

Furthermore, yield in the drought treatment was positively related to the difference in nitrogen concentration (ΔN) between the well-watered (WW N) and drought treatment (DR N) at R4 and late R5 (Table 3.10). Regressions had r^2 values of 0.47 and 0.39 for R4 and late R5 stages, respectively. At the R2 stage the relationship between yield in the DR treatment and ΔN was negative. More research is needed to elucidate why at R2 the relationships were opposite of that at other stages.

In our data, genotypes with low WW N, and WW U, and with high ΔU, and ΔN had a benefit of yield under severe drought conditions. It has been previously reported that ΔN was inversely related to WW N (King and Purcell, 2006). Since shoot nitrogen and ureide concentration are correlated (King and Purcell, 2005; Hwang et al. 2013; King et al. 2013), genotypes with low WW N will likely have low WW U and high ΔN and ΔU. In our data, genotypes with these characteristics showed an increase in yield under drought conditions. This yield increase under drought, attributable to low WW N and low WW U, has not been previously reported.

Furthermore, we found that genotypes with low WW U over the season had the highest biomass accumulation rates under drought conditions (Figure 3.8). The regression had a negative slope and an r^2 of 0.77. This relationship also has not been reported previously.

Fayetteville 2012

In the Fayetteville 2012 growing season average temperature was 24.3°C. Also, resembling 2011, mean daily temperature decreased markedly after R4 growth stage (Figure 3.9).

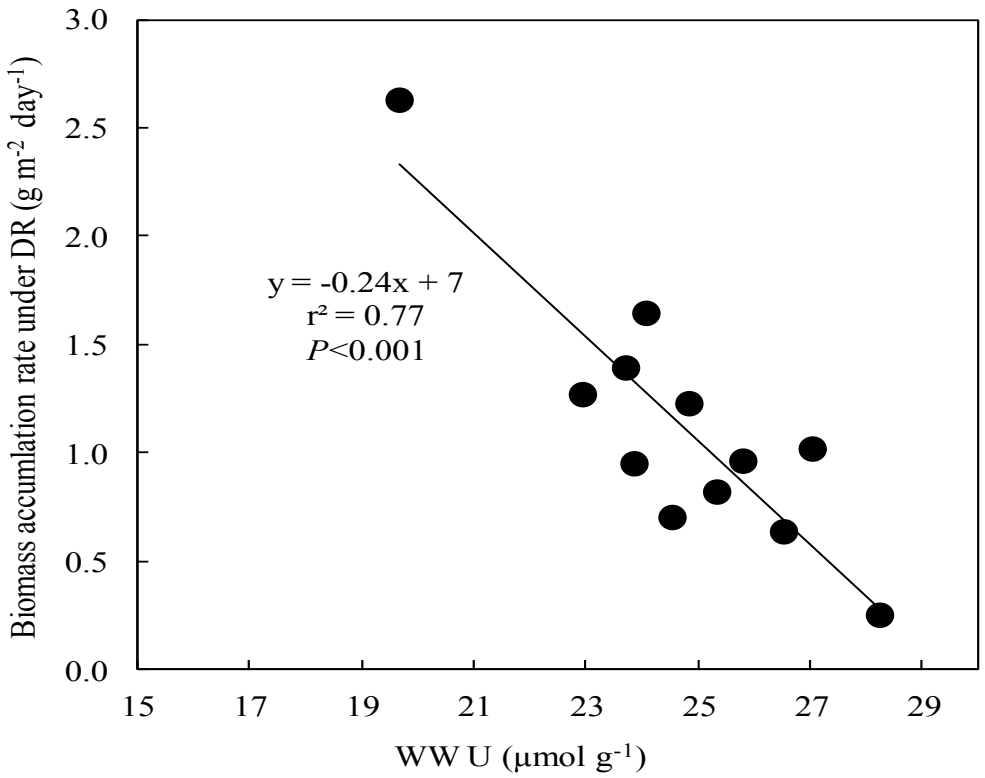

Figure 3.8. Biomass accumulation rate in the drought treatment (DR) in the V8-R3 period in Fayetteville 2011 versus shoot ureide concentration in the well-watered treatment (WW U) averaged over all sample dates. Each data point represents one genotype and is the average over 4 replications.

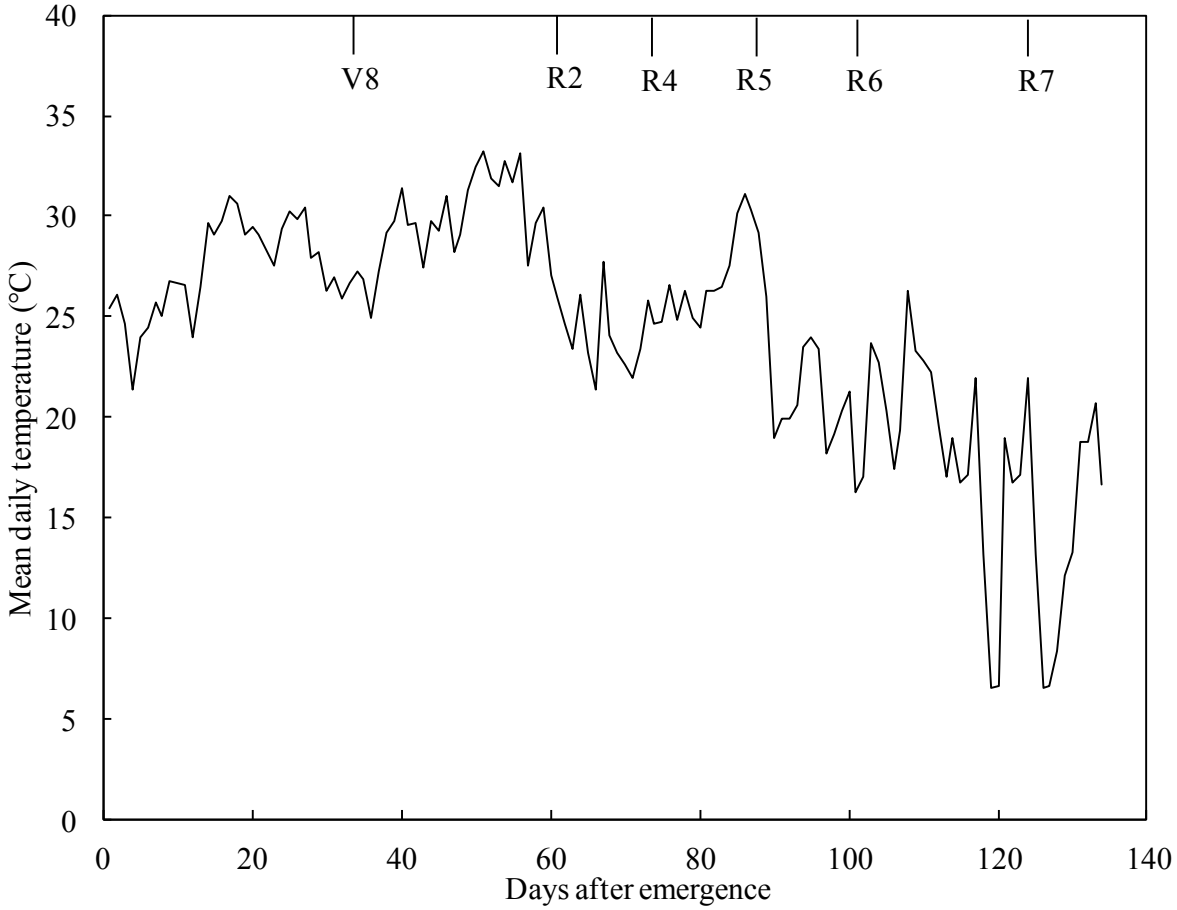

Figure 3.9. Mean daily temperature of Fayetteville 2012 versus days after emergence. Emergence was on 6 June.

In the field experiment in 2011 the drought treatment was severe, and this resulted in negative nitrogen fixation rates under drought (Figure 3.5 A). In order to avoid this, and to detect differences in nitrogen fixation among genotypes, in Fayetteville 2012 we targeted a less severe drought treatment. In Fayetteville 2012 the mean volumetric water content in the DR treatment was 0.19 cm^3cm^{-3} while in Fayetteville 2011 it was 0.14 cm^3cm^{-3}. Mean volumetric water content in the well-watered treatment was 0.24 cm^3cm^{-3} for both years.

Also, in 2012, the biomass harvest period was from R2 to R5 as compared from V8 to R3 in 2011 (Figure 3.10). Sampling time was changed to include the phenological stages at which phenotypic data for QTL analysis were collected. This late sample increased the probability of a large rainfall eliminating the drought effect, but 2012 was an especially dry year and no significant rainfall occurred during the period.

Shoot ureide and nitrogen concentration

Shoot ureide concentration was affected by water treatment, phenological stage, and genotype, and the interaction among the three factors was significant (Appendix table K). Since the three-way interaction was significant, we investigated at each developmental stage the effects and interactions of water and genotype treatments (Appendix table L).At R2 and R5 stages, there was no interaction between water treatment and genotype. When the interaction was not significant, means were separated and evaluated for the main effects as appropriate. Since water treatment had two levels (WW and DR), differences between water treatments were determined by an F-test. Shoot ureide concentration was higher in the DR treatment than in the WW treatment at R2 and was no different among water treatments at R5 (Figure 3.11).

For shoot nitrogen concentration there was interaction between water treatment and developmental stage, and the genotype main effect was significant (Appendix table K). Since the

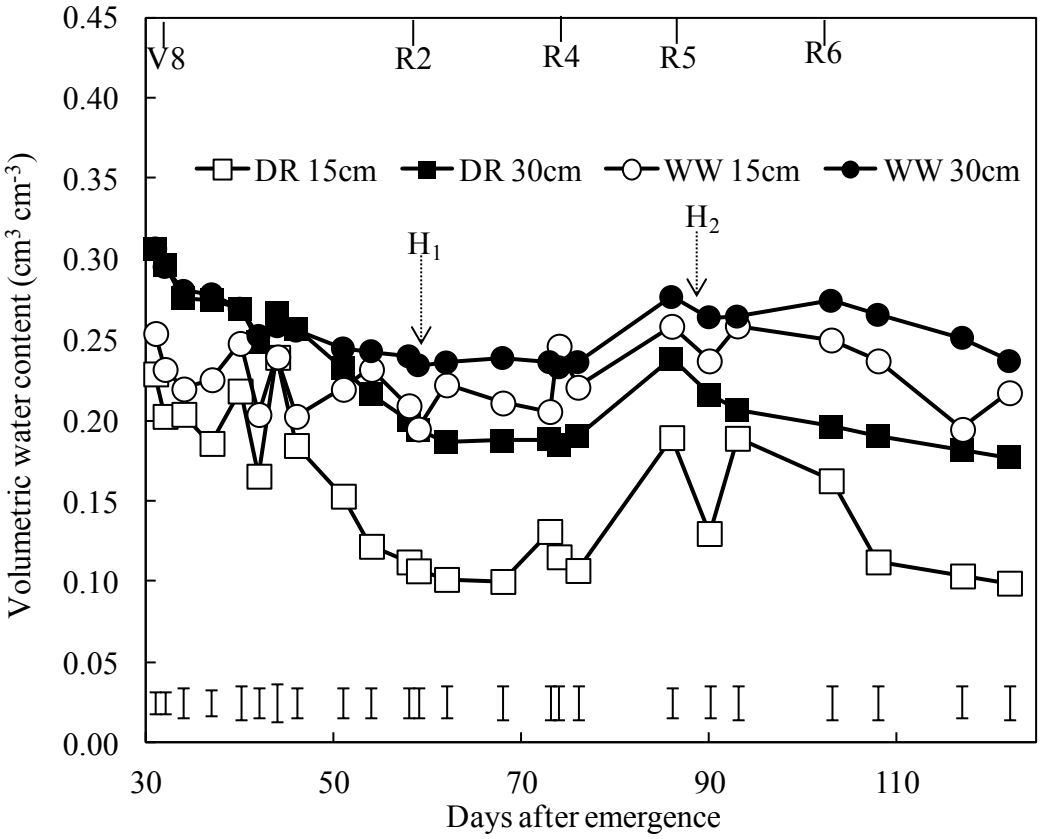

Figure 3.10. Volumetric water content ($cm^3\ cm^{-3}$) at two depths (15 cm and 30 cm) in the well-watered (WW) and drought (DR) treatments in Fayetteville 2012. Each data point is the mean of 4 replications and bars represent standard errors averaged across depths and water treatments. Biomass harvest 1 (H_1), harvest 2 (H_2), and significant phenological stages are indicated near the top of the figure.

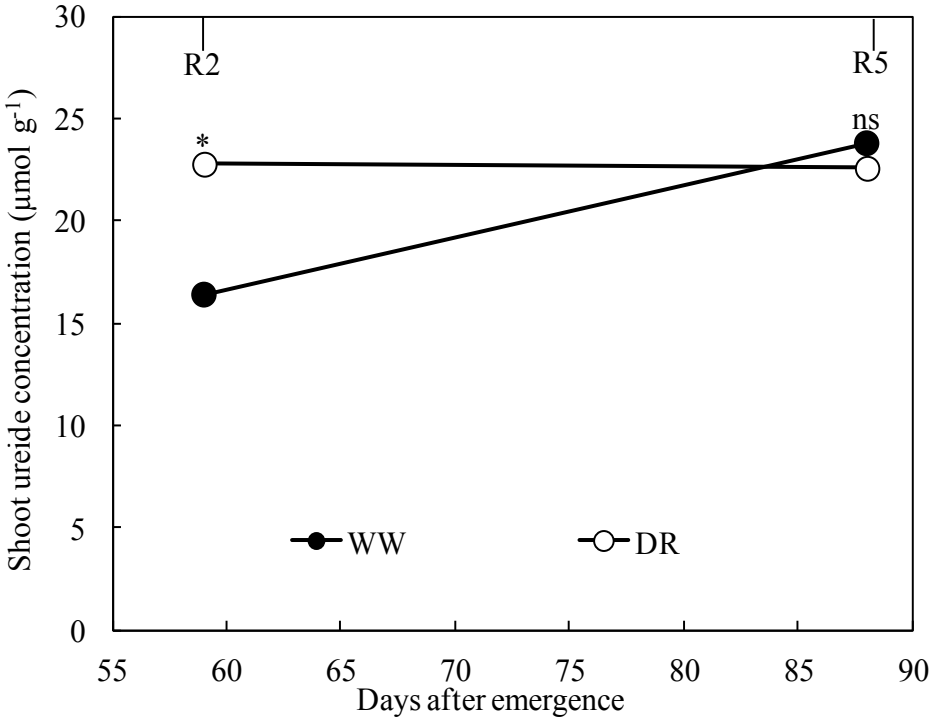

Figure 3.11. Shoot ureide concentration in the drought (DR) and well-watered (WW) treatments averaged over genotypes in Fayetteville 2012. Significant differences (*) were detected using an F-test (P<0.05) and displayed in the figure along with non significant (ns) differences.

interaction was significant we investigated at each developmental stage the effect of water treatment (Appendix table L). Shoot nitrogen concentration decreased steadily from R2 to R5 at both water treatments, and at R2 and R4 shoot nitrogen concentration was higher in the DR treatment than in the WW treatment (Figure 3.12).

A summary of the sources of variation for the responses of shoot ureide and nitrogen concentration versus the various cumulative additive effects are given in the appendix (Appendix tables M to R). In Fayetteville 2012, shoot ureide concentration under drought showed no relationship with ureide additive effects for drought conditions (Ur_CIM_{dr}) (Appendix table M). Shoot ureide concentration under well-watered and drought conditions, had significant linear and quadratic relationships with Ur_CIM_{ww} and Ur_MIM_{ww} at R2, R4, and R5 (Appendix tables M and O). Shoot ureide concentration responses at R2 to the additive effects Ur_CIM_{ww} (Figure 3.13) and Ur_MIM_{ww} (Figure 3.14) were similar under well watered and drought conditions (Figures 3.13 and 3.14). In general, the slope of the quadratic model was close to 0 for additive effects values below 0 and had a curvature up (positive slope) as the cumulative additive effect became positive. Ureide additive effects detected under WW conditions (Ur_CIM_{ww} and Ur_MIM_{ww}) explained from 22 to 60% of the variability in shoot ureide concentration (Table 3.11, Figure 3.13 and 3.14). Considering that the broad-sense heritability of shoot ureide concentration reported by Hwang et al. (2013) was 73%, the additive effects explained a large proportion of the phenotypic variability and can be useful in selecting genotypes with low ureide concentration under well-watered conditions.

Shoot nitrogen concentration under drought showed no relationship with nitrogen additive effects for drought conditions (N_CIM_{dr}) at R4 and R5, but there was a significant relationship at R2 (Appendix table Q). Shoot nitrogen concentration under well-watered and

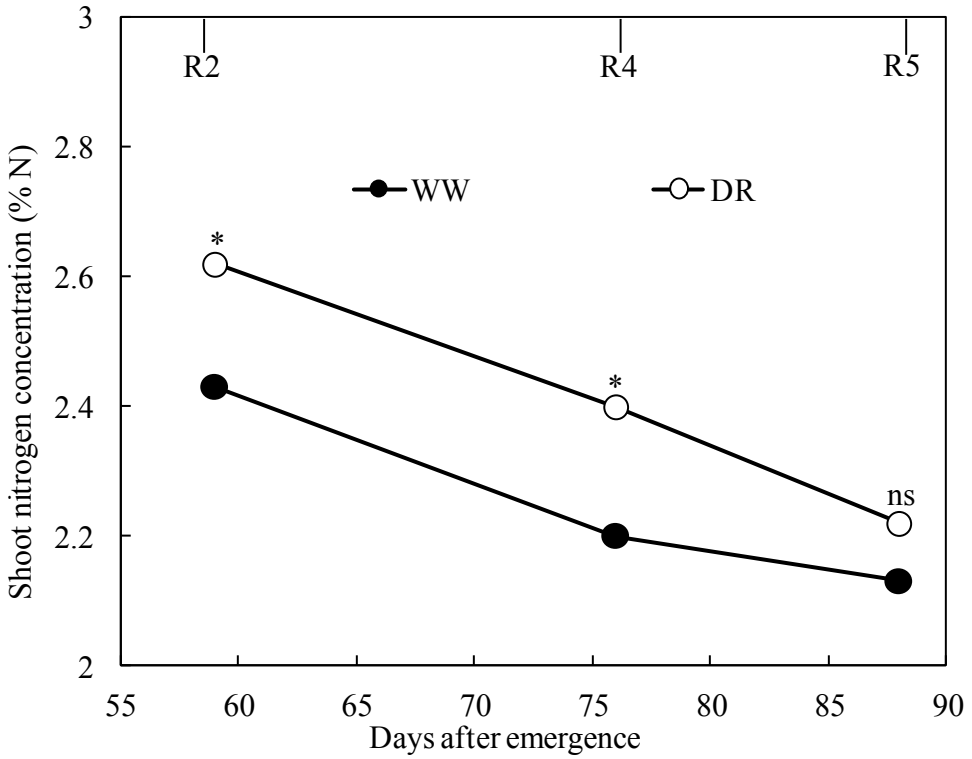

Figure 3.12. Shoot nitrogen concentration in the drought (DR) and well-watered (WW) treatments averaged over genotypes in Fayetteville 2012. Significant differences (*) were detected using an F-test ($P<0.05$) and displayed in the figure along with non significant (ns) differences.

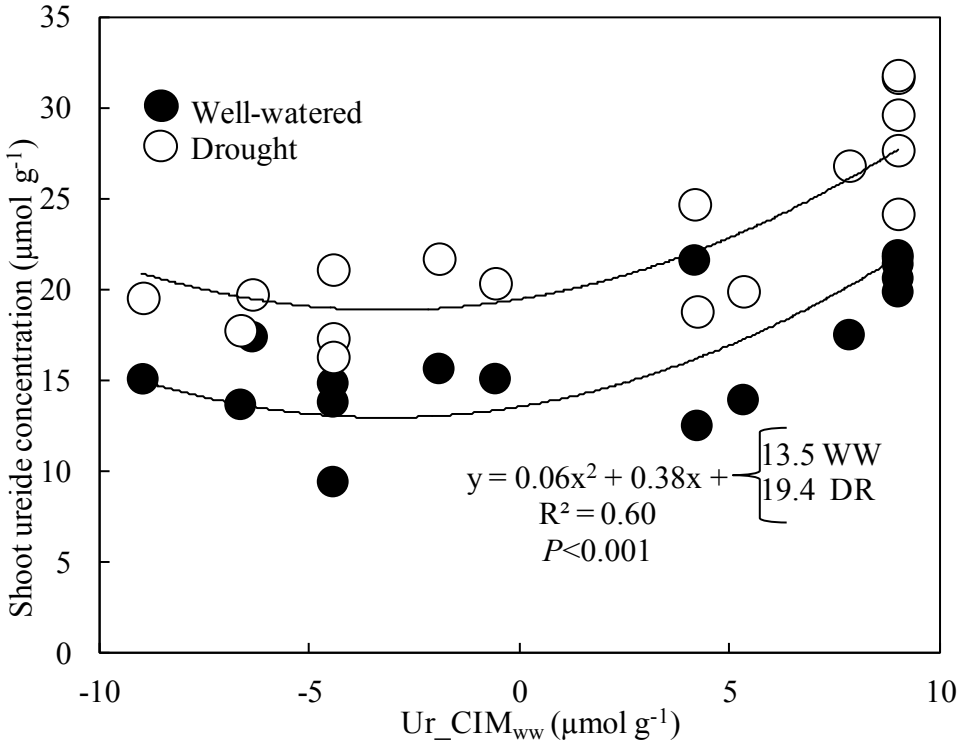

Figure 3.13. Shoot ureide concentration in Fayetteville 2012 at R2 versus the cumulative additive effects detected for well watered conditions by composite interval mapping (Ur_CIM_{ww}). Well-watered (WW) and drought (DR) treatments were considered covariates in the analysis, and the analysis indicated similar quadratic and linear coefficients for the WW and DR treatments but different intercepts. Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-values, and R^2 values were calculated using raw data.

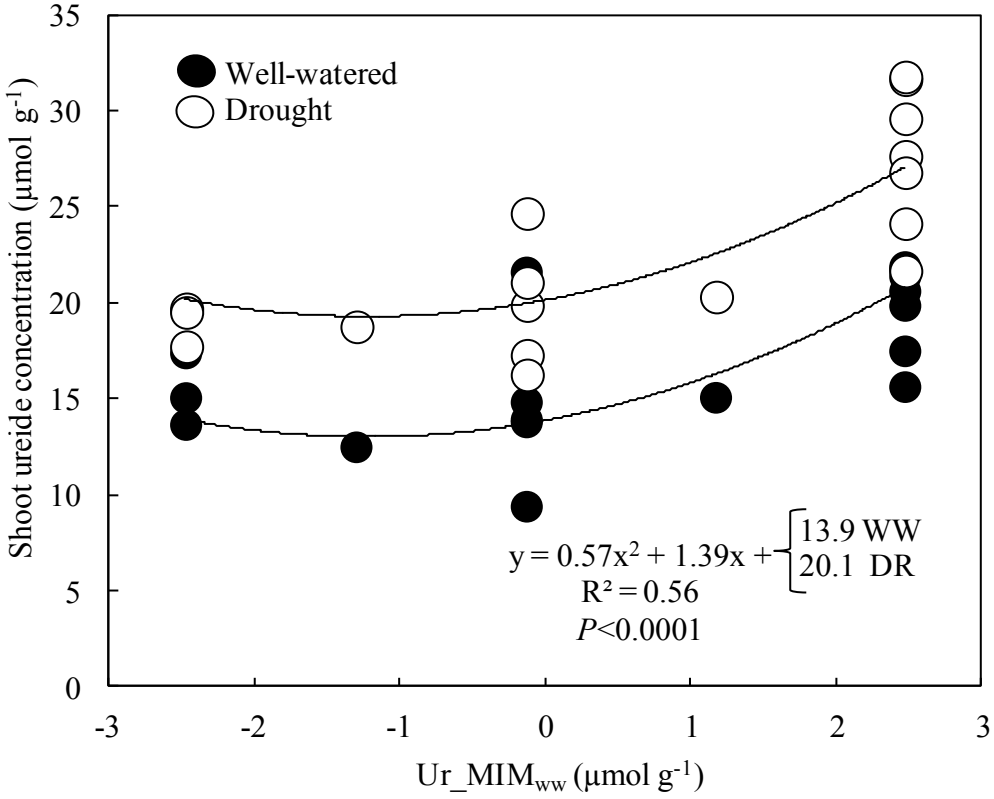

Figure 3.14. Shoot ureide concentration in Fayetteville 2012 at R2 versus the cumulative additive effects detected for well watered conditions by multiple interval mapping (Ur_MIM_{ww}). Well-watered (WW) and drought (DR) treatments were considered covariates in the analysis, and the analysis indicated similar quadratic and linear coefficients for the WW and DR treatments but different intercepts. Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-values, and R^2 values were calculated using raw data.

Table 3.11. Shoot ureide concentration in Fayetteville 2012 versus ureide additive effects detected: under well watered (WW) conditions by composite interval mapping (Ur_CIM_{ww}) and by multiple interval mapping (Ur_MIM_{ww}). Water treatment as a covariate was included when significant, otherwise water treatments were analyzed together using linear regression.

Additive effect	Dev. stage	Water trt	Parameter estimates†			R^2	Model significance
			β_0	β_1	β_2		
Ur_CIM_{ww}	R2	WW	13.5***	0.38***	0.060***	0.60	<0.0001
		DR	19.4***	0.38***	0.060***	0.60	<0.0001
	R4	WW	11.2***	0.30***	0.032*	0.38	<0.0001
		DR	15.6***	0.30***	0.032*	0.38	<0.0001
	R5	both‡	20.3***	0.62***	0.040*	0.41	<0.0001
Ur_MIM_{ww}	R2	WW	13.9***	1.39***	0.570**	0.56	<0.0001
		DR	20.1***	1.39***	0.570**	0.56	<0.0001
	R4	WW	11.5***	0.87**	0.317*	0.31	<0.0001
		DR	15.9***	0.87**	0.317*	0.31	<0.0001
	R5	both	22.0***	2.10***		0.28	<0.0001

† Quadratic responses ($y= \beta_0+\beta_1 x+\beta_2 x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

DR = drought treatment.

‡ Values of both water treatments are presented when water treatment was not significant.

drought conditions, had significant linear and quadratic relationships with N_CIM_{ww} at R2, R4, and R5, and with N_MIM_{ww} at R2 and R5 (Appendix table P and R). Nitrogen concentration at various developmental stages had significant relationships with N_CIM_{ww}, N_CIM_{dr}, and N_MIM_{ww} with r^2 values ranging from 0.09 to 0.29 (Table 3.12). Linear regressions had the same slope under well-watered and drought conditions, showing that shoot nitrogen concentration response to the additive effects was similar under both conditions. Hwang et al. (2013) reported that the broad sense heritability of shoot nitrogen concentration was 60%. Although the heritability of shoot nitrogen concentration may be lower than for shoot ureide concentration, nitrogen concentration was found not to be as dynamic and responsive to growth stage and environmental conditions as was shoot ureide concentration (Appendix table K, table 3.12). This was previously discussed by King et al. (2013).

Nitrogen Fixation and Yield

Nitrogen fixation rate (mg N m^{-2} day^{-1}) and grain yield (g m^{-2}) in Fayetteville 2012 was affected by genotype but not by water treatment (Appendix tables S and T). Also, there was no significant interaction between genotype and water treatment. The mild drought treatment apparently was not severe enough for creating significant differences between water treatments. We evaluated nitrogen fixation and yield responses as a function of the additive effects with water treatment as a covariate factor (Appendix tables U, V, and W).

Water treatments and additive effects for Ur_CIM_{ww}, Ur_MIM, and N_CIM_{ww} explained 20%, 23%, and 17% of the variability in nitrogen fixation rate, respectively (Table 3.13). Genotypes with alleles for high ureide concentration showed higher nitrogen fixation rates under WW conditions than genotypes with alleles for low ureide concentration (Figure 3.16). This can be expected since ureides are the product of nitrogen fixation and high nitrogen fixation rates

Table 3.12 . Shoot nitrogen concentration in Fayetteville 2012 versus nitrogen additive effects detected: under well watered (WW) conditions by composite interval mapping (N_CIM_{ww}) and by multiple interval mapping (N_MIM_{ww}). Water treatment as a covariate was included when significant, otherwise water treatments were analyzed together using linear regression.

Additive effect	Dev. stage	Water trt	Parameter estimates†			R^2	Model significance
			β_0	β_1	β_2		
N_CIM_{ww}	R2	WW	2.42***	0.98***		0.29	<0.0001
		DR	2.61***	0.98***		0.29	<0.0001
	R4	WW	2.11***	0.65**	4.06**	0.24	<0.0001
		DR	2.32***	-0.01^{ns}	4.06**	0.24	<0.0001
	R5	both‡	2.10***	0.71***	2.81*	0.20	<0.0001
N_CIM_{dr}	R2	DR	2.64***	3.44**		0.19	<0.01
N_MIM_{ww}	R2	WW	2.38***	2.00**		0.20	<0.0001
		DR	2.60***	2.00**		0.20	<0.0001
	R5	WW	2.09***	1.75**		0.09	<0.01
		DR	2.20***	1.75**		0.09	<0.01

† Quadratic responses ($y= \beta_0+\beta_1 x+\beta_2 x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

DR = drought treatment.

‡ Values of both water treatments are presented when water treatment was not significant.

Table 3.13. Nitrogen fixation rate (mg N m^{-2} day^{-1}) and yield (g m^{-2}) in Fayetteville 2012 versus: ureide cumulative additive effects for well-watered (WW) conditions detected by composite interval mapping (Ur_CIM_{ww}), ureide cumulative additive effects for well-watered conditions detected by multiple interval mapping (Ur_MIM_{ww}), and nitrogen cumulative additive effects for well-watered conditions detected by composite interval mapping (N_CIM_{ww}). Water treatment was used as a covariate in the analysis.

Dependent variable	Independent variable	Water treatment	Parameter estimates		R^2	Model significance
			β_0	β_1		
N_2 fixation rate	Ur_CIM_{ww}	WW	168***	5.0**	0.20	0.0002
		DR	127***	-1.8^{ns}	0.20	0.0002
	Ur_MIM_{ww}	WW	163***	19.5**	0.23	<0.0001
		DR	127***	-6.2^{ns}	0.23	<0.0001
	N_CIM_{ww}	WW	174***	218.0*	0.17	0.0008
		DR	125***	-77.0^{ns}	0.17	0.0008
Yield	Ur_CIM_{ww}	WW	232***	4.1***	0.22	<0.0001
		DR	201***	1.3^{ns}	0.22	<0.0001
	Ur_MIM_{ww}	WW	232***	13.4***	0.23	<0.0001
		DR	198***	7.6*	0.23	<0.0001
	N_CIM_{ww}	WW	236***	198***	0.22	<0.0001
		DR	202***	62.1^{ns}	0.22	<0.0001

DR = drought treatment.

Ns= no significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

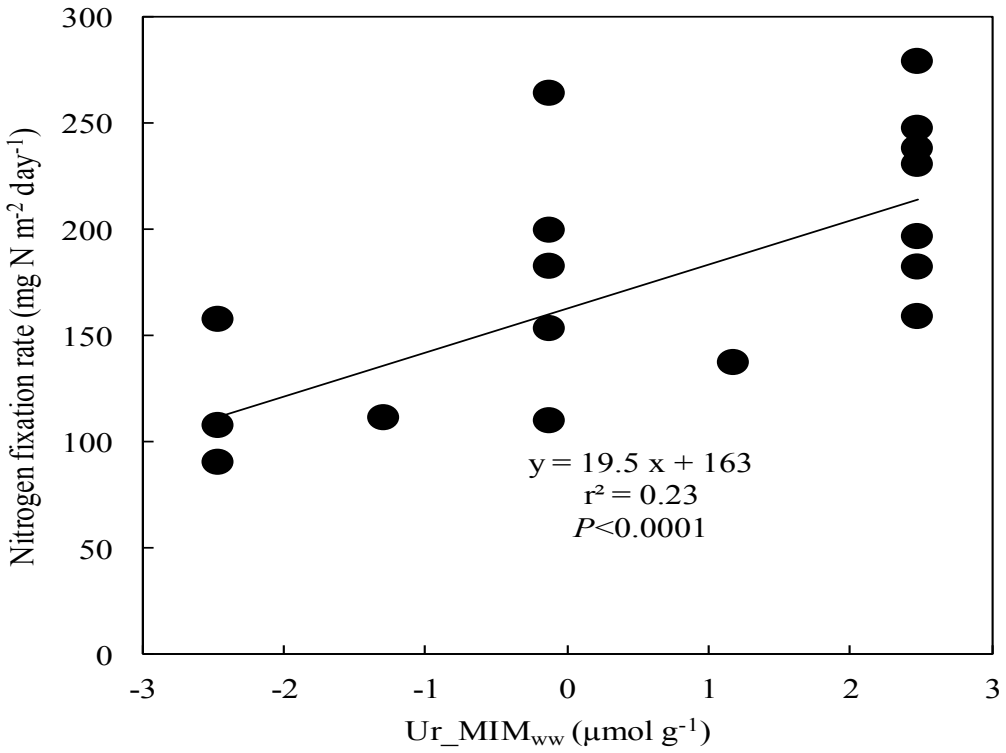

Figure 3.16. Nitrogen fixation rate under well-watered conditions in the R2-R5 period in Fayetteville 2012, versus ureide additive effects detected by multiple interval mapping (Ur_MIM_{ww}). Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-value, and r^2 were calculated using raw data.

resulted in high shoot ureide concentration. Further, ureide concentration in the xylem sap and leaf tissue has been extensively used as a method for measuring nitrogen fixation (Peoples et al., 2009).

In the well-watered treatment nitrogen fixation rate ranged from 90 to 279 mg N m^{-2} day^{-1} and represented 49.4 and 66.8% of the total shoot nitrogen at R2 and R5, respectively. Nitrogen fixation rates were low compared with reports in the literature (Mastrodomenico and Purcell, 2012). This low nitrogen fixation rate may be a result of 71 kg ha^{-1} of soil inorganic nitrogen at emergence in 2012. Inorganic nitrogen is known to inhibit nitrogen fixation (Allos and Bartholomew, 1959). The source of this residual nitrogen is unknown, since the previous rye crop would expectantly removed most of the soil nitrogen.

Under drought, the slopes of the regressions for nitrogen fixation rate versus the various cumulative additive effects were negative but not significant (Table 3.13). This shows that nitrogen fixation under DR was affected by water treatment but not by the cumulative additive effects. Nitrogen fixation accounted for 35.5% and 55% of the total shoot nitrogen at R2 and R5, respectively.

Yield under WW conditions was positively associated with Ur_CIM_{ww}, Ur_MIM, and N_CIM_{ww} (Table 3.13). The R^2 values of the linear regressions between WW yield and the additive effects were 0.22 and 0.23, slopes were highly significant ($P<0.0001$). Under WW conditions, genotypes with alleles for high ureide and nitrogen concentration had higher yield than genotypes with alleles for low ureide and nitrogen concentration (Figure 3.17). Under drought conditions, in 2 out of 3 regressions, the slopes were not significant (Table 3.13). In the regression in which the additive effects significantly affected yield under DR, the *P*-value and

the slope were lower than under WW conditions. Consequently, under DR conditions the additive effects did not affect yield as they did under WW conditions.

Keiser 2012

In the Keiser field experiment, the mean daily temperature was 24.4°C and decreased markedly after R4 as in Fayetteville 2011 and 2012 (Figure 3.18). Drought and well watered treatments received the same irrigation until 55 days after planting when the drought treatment was initiated (Figure 3.19). Rainfalls events occurred during the season and as a consequence the drought effect was reduced at R2 and eliminated at R4.

Shoot ureide and nitrogen concentration

Shoot ureide and nitrogen concentrations were affected by phenological stage, and genotype, and the interaction between these factors was significant (Appendix table X). Drought treatment was included at R2 only, when shoot ureide and nitrogen concentrations were not different under well watered and mild drought conditions (Appendix table Y). The genotype response was analyzed as described for Fayetteville by using the cumulative additive effects of ureide or nitrogen concentrations for each genotype. Simple linear and quadratic regressions were determined, using the cumulative additive effects as independent variables and shoot ureide and nitrogen concentration as dependent variables (Appendix tables Z and AA). A summary of the sources of variation for the responses of shoot ureide and nitrogen concentration versus the various cumulative additive effects are given in the appendix (Appendix tables AB, AC, AD, and AE).

For Keiser in 2012, ureide additive effects detected under well watered (Ur_CIM_{ww} and U_MIM_{ww}), but not under drought conditions (Ur_CIM_{dr}), were associated (linear or quadratic models) with shoot ureide concentration at all developmental stages (Appendix tables Z, AB, and

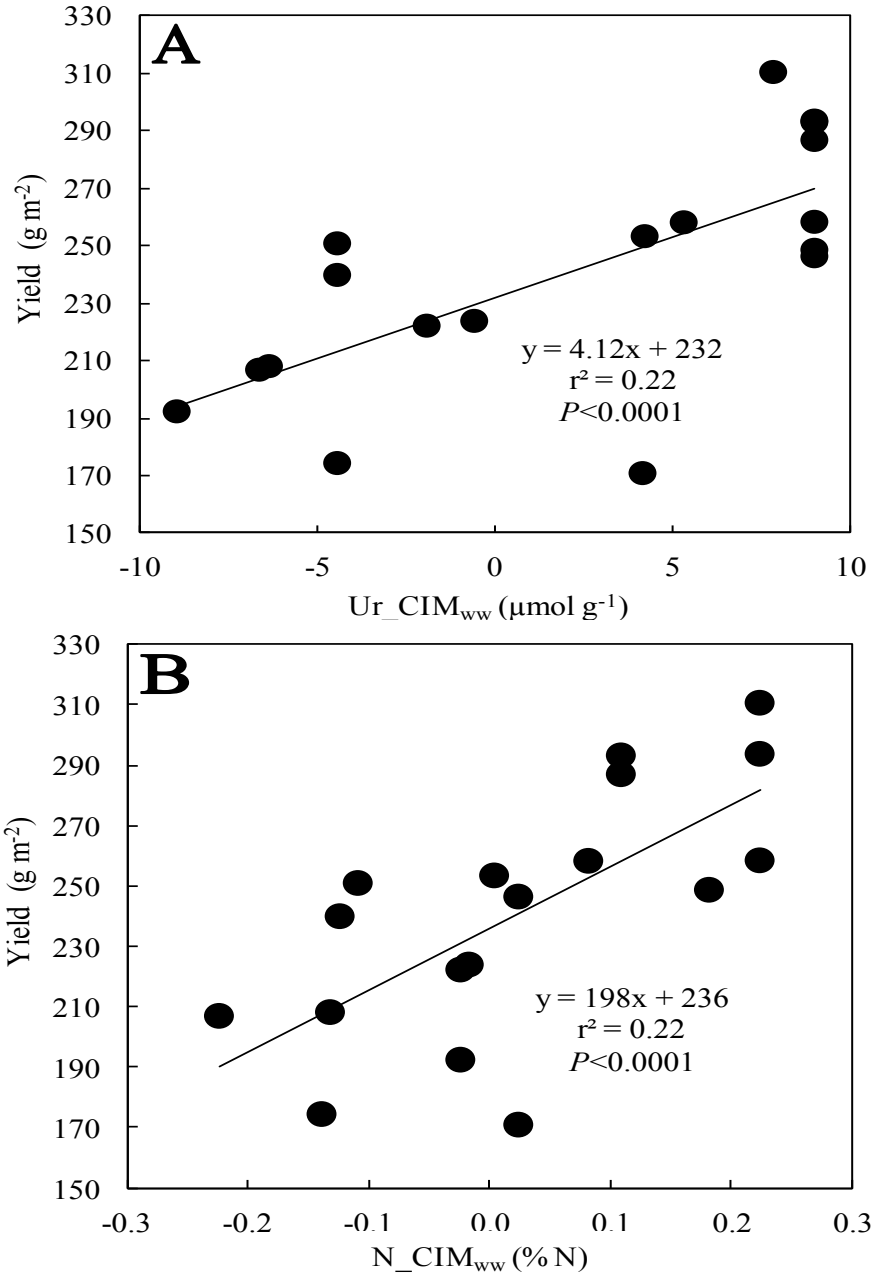

Figure 3.17. Yield under well-watered (WW) conditions in Fayetteville 2011 versus A) ureide additive effects (Ur_CIM_{ww}) and B) nitrogen additive effects (N_CIM_{ww}) detected by composite interval mapping under WW conditions. Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-values, and r^2 were calculated using raw data.

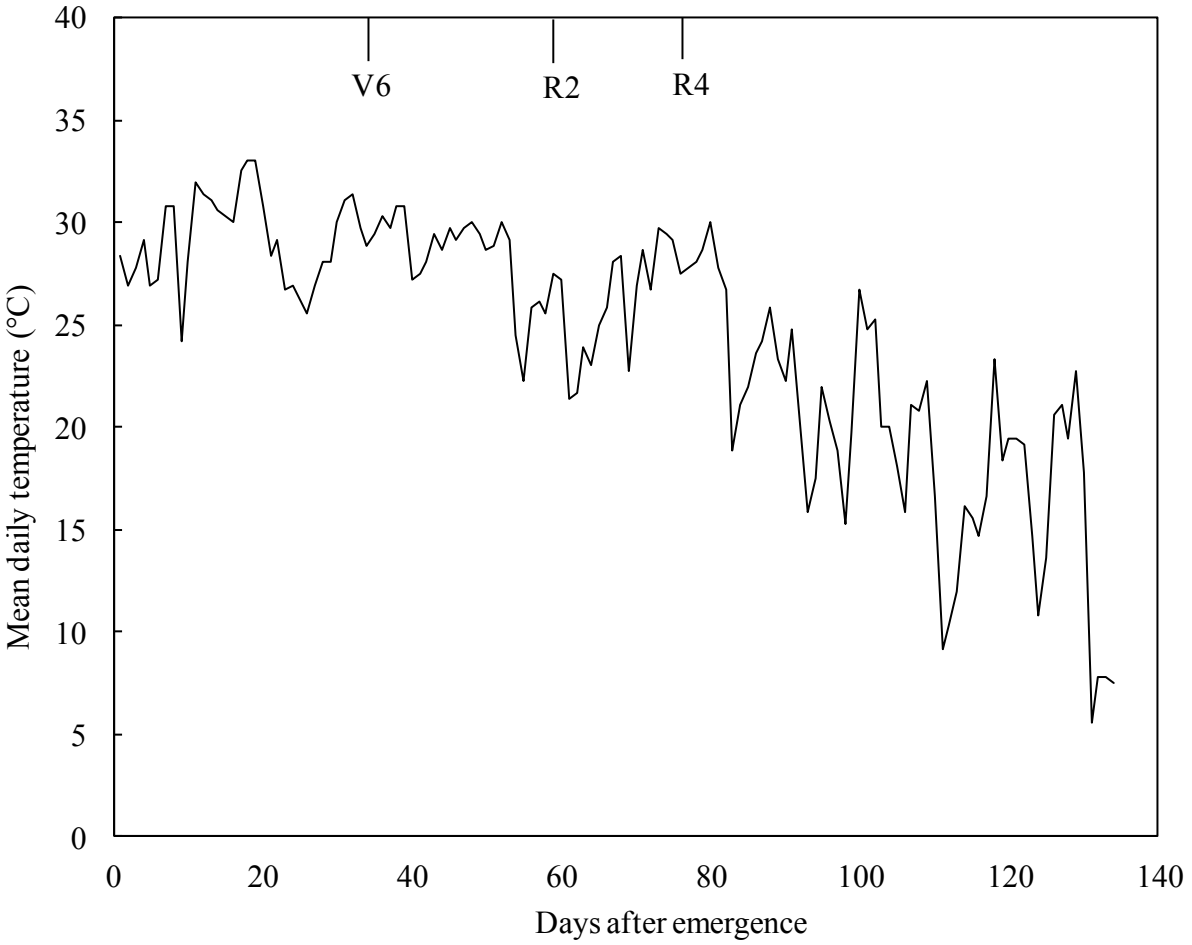

Figure 3.18. Mean daily temperature of Keiser 2012 versus days after emergence. Emergence was on 13 June.

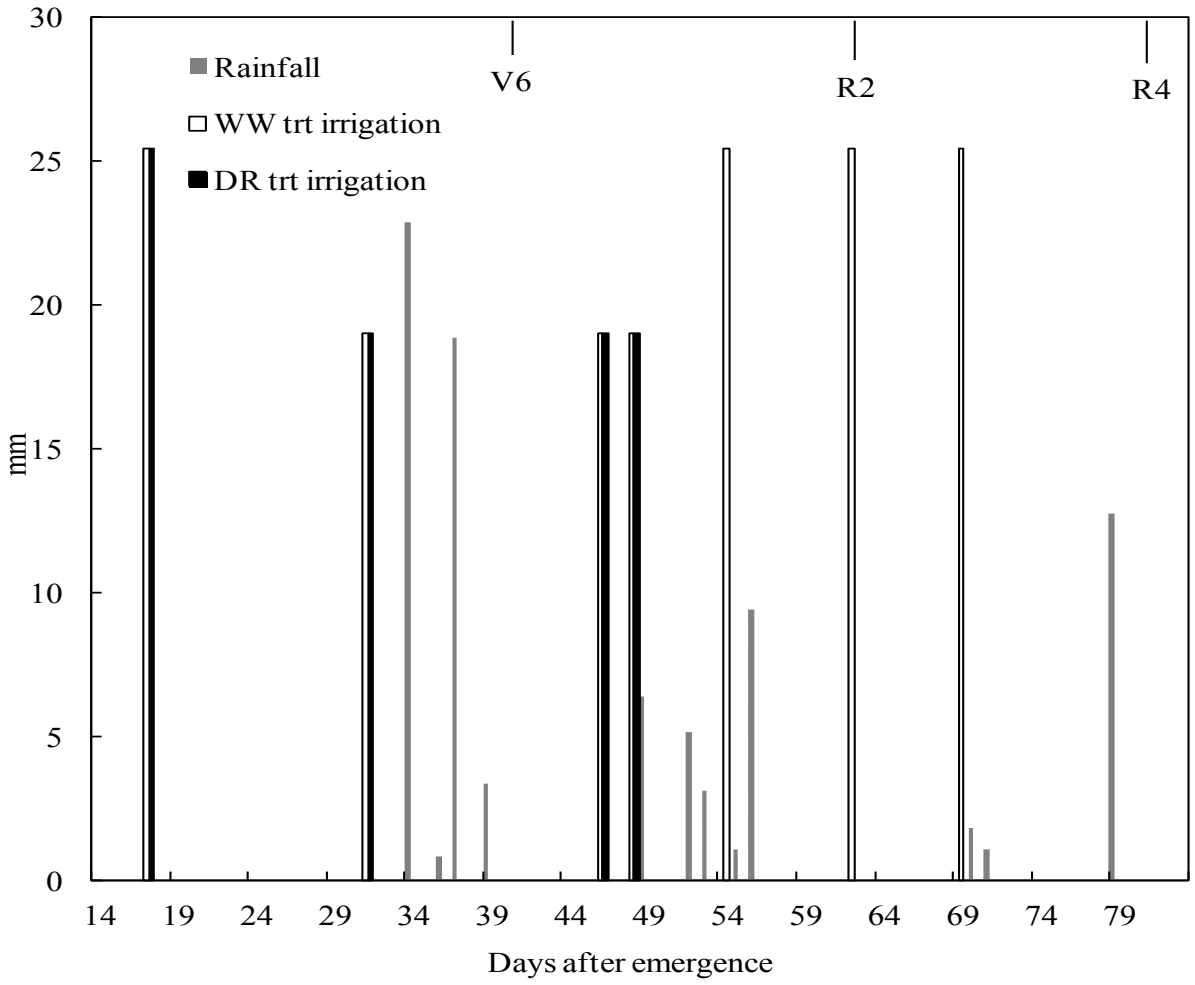

Figure 3.19. Seasonal rainfall and irrigation for the well watered (WW) and drought (DR) treatments in Keiser 2012. Significant developmental stages are indicated near the top of the figure.

AC). Ureide additive effects detected by composite interval mapping under WW conditions (Ur_CIM_{ww}) explained from 12 (V6) to 65% (R4) of the variability in shoot ureide concentration (Table 3.14). In general, the slope of the quadratic model was close to 0 for additive effects values below 0 and had a curvature up (positive slope) as the cumulative additive effect became positive (Figure 3.20). Ureide additive effects detected by multiple interval mapping (Ur_MIM_{ww}) explained from 13% to 26% of the variability in ureide concentration (Table 3.14).

Nitrogen additive effects detected by: composite interval mapping for well watered conditions (N_CIM_{ww}) and for drought conditions (N_CIM_{dr}), and by multiple interval mapping for well watered conditions (N_MIM_{ww}) were linearly associated with nitrogen concentration (Appendix tables AA, AD, and AE). Nitrogen additive effects detected by CIM under well watered conditions (N_CIM_{ww}) explained from 25 to 55% of the variability in nitrogen concentration (Table 3.15, Figure 3.21 A). Nitrogen additive effects for drought conditions (N_CIM_{dr}) explained 19% of the variability in nitrogen concentration in the DR treatment (Table 3.15). Nitrogen additive effects detected by MIM (N_MIM_{ww}) explained 9 (V6), 15 (R2), and 40% (R4) of the variability in nitrogen concentration (Figure 3.21B).

Relationship between shoot ureide and nitrogen concentration

Shoot ureide and nitrogen concentrations, when averaged over all sample dates, were significantly correlated in 5 out of 6 environments (Table 3.16). In Keiser 2012 for the DR treatment, there was no significant correlation between these variables. There was only one sample date in Keiser in the DR treatment. In Fayetteville 2011 and 2012, however, using several sample dates, under both WW and DR conditions, the correlation was significant and high ($P<0.001$, $r \geq 0.65$). This correlation was previously reported by King and Purcell (2006) and more recently by Hwang et al. (2013) and King et al. (2013).

Table 3.14. Shoot ureide concentration in Keiser 2012 versus: ureide additive effects for well-watered (WW) conditions detected by composite interval mapping (Ur_CIM_{ww}) and multiple interval mapping (Ur_MIM_{ww}). Water treatment as a covariate was included at R2. At V6 and R4, analysis was done using linear regression.

Add. effect	Dev. Stage	Water trt	Parameter estimates†			R^2	Model significance
			β_0	β_1	β_2		
Ur_CIM_{ww}	V6	WW	11.9***	-0.037^{ns}	0.028**	0.12	<0.05
	R2	both‡	15.8***	-0.001^{ns}	0.058***	0.30	<0.0001
	R4	WW	39.4***	1.067***	0.087*	0.65	<0.0001
Ur_MIM_{ww}	V6	WW	11.9***	0.022^{ns}	0.278**	0.13	<0.05
	R2	both	16.2***	0.364*	0.510***	0.30	<0.0001
	R4	WW	43.9***	2.859***		0.26	<0.0001

At V6 and R4 there was no drought treatment.

† Quadratic responses ($y= \beta_0+\beta_1 x+\beta_2 x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

‡ Values of both water treatments are presented when the water treatment effect was not significant.

Ns= no significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

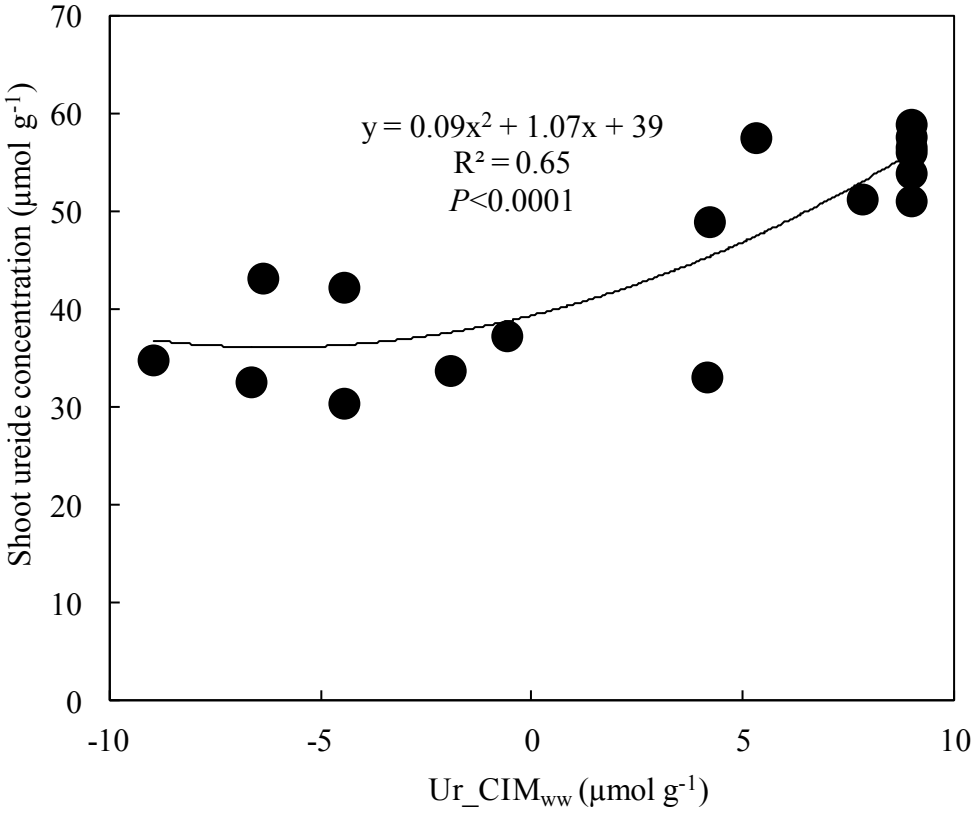

Figure 3.20. Shoot ureide concentration in Keiser 2012 at R4 stage under well-watered conditions, versus ureide cumulative additive effects for well-watered conditions detected by composite interval mapping (Ur_CIM_{ww}). Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-value, and R^2 were calculated using raw data.

Table 3.15. Shoot nitrogen concentration in Keiser 2012 versus: nitrogen cumulative additive effects for well-watered (WW) conditions detected by composite interval mapping (N_CIM_{ww}), nitrogen cumulative additive effects for drought (DR) conditions detected by composite interval mapping (N_CIM_{dr}), and nitrogen cumulative additive effects for WW conditions detected by multiple interval mapping (N_MIM_{ww}). Water treatment as a covariate was included at R2. At V6 and R4, analysis was done using linear regression.

Add. effect	Dev. Stage	Water trt	Parameter estimates†			Model significance
			β_0	β_1	r^2	
N_CIM_{ww}	R2	both‡	2.56***	0.95***	0.25	<0.0001
	R4	WW	2.76***	1.41***	0.55	<0.0001
N_CIM_{dr}	R2	DR	2.56***	3.89**	0.19	<0.001
N_MIM_{ww}	V6	WW	3.25***	1.89*	0.09	<0.05
	R2	both	2.53***	2.45***	0.15	0.0001
	R4	WW	2.71***	4.16***	0.40	<0.0001

At V6 and R4 there was no drought treatment.

† Quadratic responses ($y= \beta_0+\beta_1 x+\beta_2 x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

‡ Values for both water treatments are presented when the water treatment effect was not significant.

DR = drought treatment.

*Significant at the 0.05 probability level.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

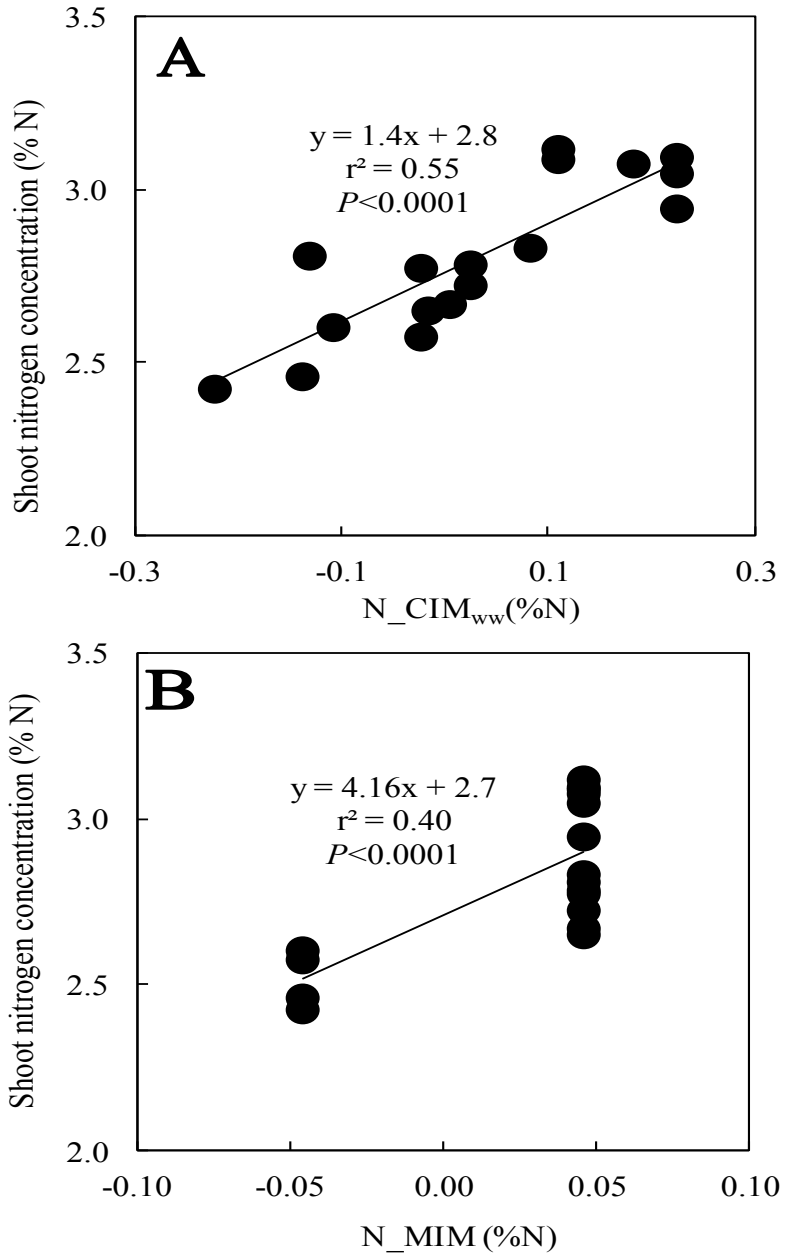

Figure 3.21. Shoot nitrogen concentration in Keiser 2012 at R4 stage under well-watered conditions, versus nitrogen additive effects for well-watered conditions detected by: A) composite interval mapping (N_CIM_{ww}) and B) multiple interval mapping (N_MIM). Each data point represents one genotype and is the average over 4 replications. Regression parameters, *P*-values, and r^2 were calculated using raw data.

Table 3.16. Pearson's coefficient of determination (r) for the correlation between shoot ureide concentration and nitrogen concentration at six environments averaged over all sample dates.

Year	Location	Water treatment	r =	*P*-value
2011	Fayettteville	WW	0.67	<0.001
2011	Fayettteville	DR	0.77	<0.001
2012	Fayettteville	WW	0.76	<0.001
2012	Fayettteville	DR	0.65	<0.001
2012	Keiser	WW	0.74	<0.001
2012	Keiser	DR	0.23	ns

Ns = non significant correlation (P>0.05).

WW = well-watered treatment.

DR = drought treatment.

Nitrogen Fixation

The percentage of nitrogen derived from the atmosphere (%Ndfa) was significantly affected by developmental stage, and there was interaction between genotype and water treatment (Appendix table AF). Using the additive effects as an independent variable and developmental stages as covariates, we investigated the relationships between cumulative additive effects and %Ndfa. Addititve effects detected by multiple interval mapping were not significantly associated with %Ndfa (Appendix tables AG, and AH). Percentage of nitrogen derived from the atmosphere (%Ndfa) was linearly associated with Ur_CIM_{ww} and there was no interaction with water treatment or developmental stage (Appendix table AH). Also, %Ndfa showed a linear response to N_CIM_{ww} and no interactions with water treatment or developmental stage (Appendix table AI). Regression coefficients (r^2) ranged from 0.40 to 0.41 (Table 3.17). The regression with the best fit was between %Ndfa and Ur_CIM_{ww}, and this relationship followed the same trend at R2 and R4 developmental stages (Figure 3.22). Percentage of nitrogen derived from the atmosphere was higher at R4 than at R2. This might be because the crop became more dependent upon nitrogen fixation as soil nitrogen was depleted in the soil. Genotypes with alleles for high ureide and nitrogen concentration under WW conditions had higher %Ndfa than genotypes with alleles for low ureide and nitrogen concentration.

Table 3.17. Nitrogen derived from the atmosphere (%Ndfa) in Keiser 2012 versus: ureide cumulative additive effects for well-watered conditions detected by composite interval mapping (Ur_CIM_{ww}), and nitrogen cumulative additive effects for well-watered conditions detected by composite interval mapping (N_CIM_{ww}). Developmental stage was used as a covariate in the analysis.

Additive effect	Dev. stage	Parameter estimates†			Model significance
		β_0	β_1	r^2	
Ur_CIM_{ww}	R2‡	77.7***	0.18**	0.41	<0.0001
Ur_CIM_{ww}	R4	84.8***	0.18**	0.41	<0.0001
N_CIM_{ww}	R2‡	78.5***	6.91**	0.40	<0.0001
N_CIM_{ww}	R4	85.6***	6.91**	0.40	<0.0001

† Quadratic responses ($y= \beta_0+\beta_1x+\beta_2x^2$) were determined when they were significant; otherwise, only linear coefficients are reported.

‡At R2 stage, data from the DR treatment were considered under WW conditions due to absence of water treatment effect or interaction between water treatment and additive effects.

**Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

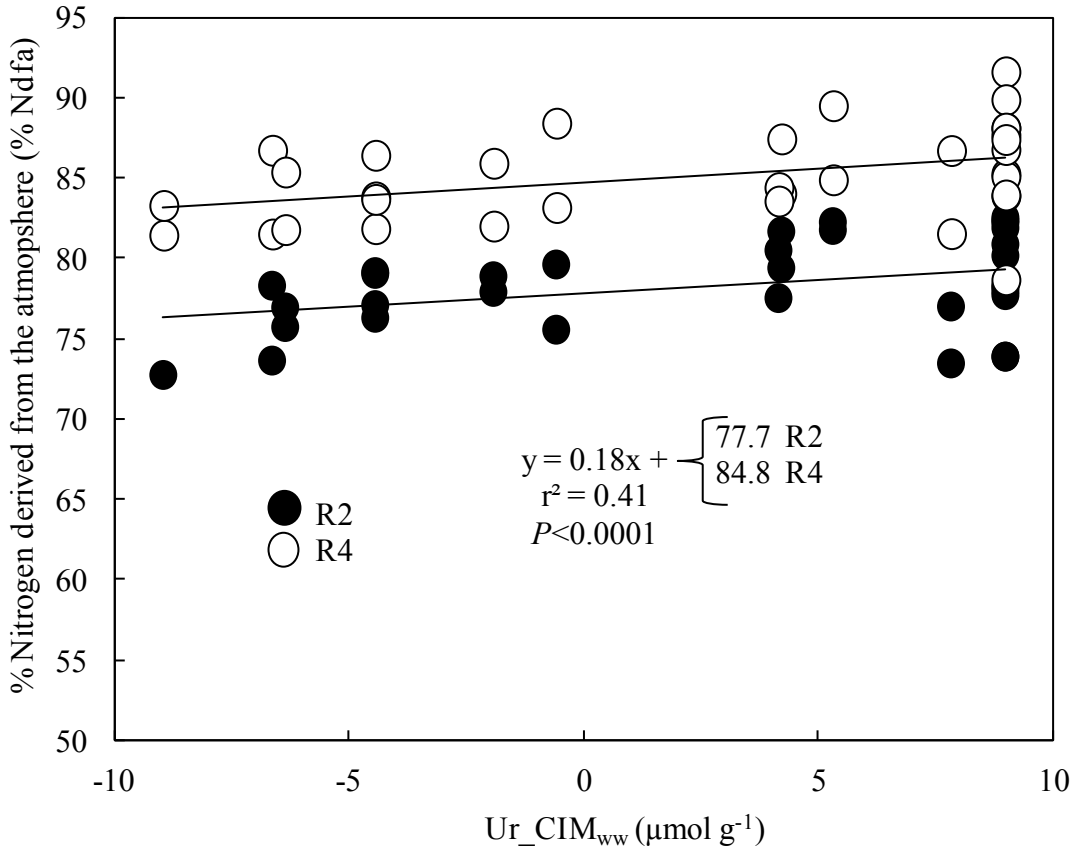

Figure 3.22. Percentage of nitrogen derived from the atmosphere (%Ndfa) at R2 and R4 at Keiser 2012, versus ureide additive effects for well-watered conditions detected by composite interval mapping (Ur_CIM$_{ww}$). Each data point represents one genotype and is the average over 4 replications. Data from the DR treatment were considered to be under well-watered conditions since there was no drought effect or interaction between water treatment and additive effects. Developmental stages were considered covariates in the analysis, and the analysis indicated similar linear coefficients for R2 and R4 developmental stages but different intercepts.

Discussion and Conclusions

In the field experiment in 2011, our data show that under severe drought as ΔU increased nitrogen fixation rate increased (Figure 3.6). Similarly, as WW U and WW N decreased, yield under drought increased (Figure 3.7). King and Purcell (2006) demonstrated that genotypes with low WW N had superior nitrogen accumulation under drought. More recently, King et al. (2013) showed that genotypes with low WW N continued to fix nitrogen under drier soil conditions than genotypes with high WW N. However, an increase in growth and yield under drought, associated with low WW shoot ureide and nitrogen concentrations, has not been previously reported.

In 2012, drought stress severity at both Fayetteville and Keiser was less than in 2011 at Fayetteville. In Fayetteville 2012, under well watered conditions, genotypes with alleles for increased ureide and nitrogen concentration had a higher nitrogen fixation rate (Figure 3.16) and grain yield (Figure 3.17) than genotypes with negative additive effects. In Keiser, under well-watered conditions, genotypes with alleles for high ureide and nitrogen concentration had higher %Ndfa, at R2 and R4 stages, than genotypes with alleles for low ureide and nitrogen concentration (Table 3.17, figure 3.22).

The relatively low proportion of the variability in nitrogen fixation rate, %Ndfa, and yield, explained by the cumulative additive effects was expected as nitrogen fixation ability and yield are traits affected by multiple genes and with low heritability. Ronis et al. (1985) reported broad sense heritability ranging from 0.53 to 0.60 for the amount of seed N derived from N_2 fixation, and Johnson et al. (1955), and later Anand and Torrie (1963) reported heritabilities of grain yield less than 0.50.

Our data suggest that under well watered conditions, genotypes with alleles for increased ureide and nitrogen concentration will likely have higher yield. However, there is controversy

about the relationship between shoot nitrogen concentration and seed yield under well watered conditions. Pal and Saxena (1976) found a positive correlation between yield and leaf nitrogen concentration at R2 and R5, while Shibles and Sundberg (1998) found a poor relationship between both traits. Jeppson et al. (1978) reported differences in total shoot N among similar yielding genotypes. King et al. (2013) found no correlations and moderate negative correlations between shoot nitrogen concentration, at R2 and R5 developmental stages, and seed yield in four environments. Previous reports suggest that genotypes with low WW N, low WW U, and high yield under well watered conditions can be selected. These genotypes can be tested under drought conditions and incorporated as drought tolerant parents in breeding programs.

The QTLs detected by Hwang et al. (2013) predicted well the phenotypes in 2012. In 2011, RILs were selected using preliminary QTL data and did not have extreme values for additive effects. The second year the additive effects and the phenotype matched well, especially at Keiser. In both years, at most of the developmental stages, the interactions between the additive effects and water treatment were not significant, showing that QTLs detected under well watered conditions can be used under drought conditions as well.

Shoot ureide and nitrogen concentrations were found to be predicted by the cumulative additive effects. Hwang et al. (2013) reported a heritability of 0.73 and 0.60, under well-watered conditions, for shoot ureide and nitrogen concentration, respectively. Stability of the trait among developmental stages and environments must be considered when choosing a trait for selection. Although the heritability of nitrogen concentration may be lower than the heritability of ureide concentration, genotypic differences in nitrogen concentration were found to be stable across phenological stages and water treatments (Figure 3.3, 3.12, Appendix table K). King et al. (2013) comparing shoot nitrogen and ureide concentration among 22 plant introductions

concluded that nitrogen concentration was more stable than ureide concentration, although changes in nitrogen concentration across developmental stages should be considered.

Shoot nitrogen concentration and shoot ureide concentration were found to be correlated in 5 out of 6 environments (Table 3.16). This agrees with previous reports by King and Purcell (2006), Hwang et al. (2013), and King et al. (2013). Because these variables are highly correlated, selection based on either low %N or low ureide concentration will likely select the same genotypes.

Our data provide evidence that selection for low WW N and low WW U may be useful for identifying genotypes with superior nitrogen fixation, growth, and yield under drought conditions. Since nitrogen and ureide concentration may be positively correlated with yield under well watered conditions, genotypes with high yield and low WW N and WW U shoot concentrations need to be identified. The QTLs detected by Hwang et al. (2013) are an important tool for selecting these phenotypes.

In addition, the QTLs can be used for introgression of drought tolerance genes into high yielding cultivars. RILs with alleles for low WW N and WW U can be backcrossed with high yielding genotypes and the resulting progeny can be selected using the molecular markers associated with shoot ureide or nitrogen concentration reported by Hwang et al. (2013). The use of marker assisted selection in backcrossing programs provides a means of efficiently incorporating this trait into different genetic backgrounds and to determine the value in ameliorating the effects of drought.

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V. APPENDIX

Appendix Table A. Analysis of variance (ANOVA) for shoot ureide concentration and nitrogen concentration in Fayetteville 2011.

Shoot ureide concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	9170.73	110.65	<0.0001
Stage	8	5383.39	626.32	<0.0001
Genotype	11	1691.87	9.43	<0.0001
Water treatment x stage x genotype	88	34.22	3.98	<0.0001

Shoot nitrogen concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	2.55	16.76	0.0064
Stage	8	44.08	964.08	<0.0001
Genotype	11	0.58	12.64	<0.0001
Water treatment x stage x genotype	88	0.061	1.34	0.0299

Appendix Table B. Analysis of variance by phenological stage for shoot ureide concentration and nitrogen concentration in Fayetteville 2011.

Shoot ureide concentration

	Source		
Stage	Water treatment (wtrt)	Genotype	wtrt x genotype
V5	*	***	**
V7	ns	***	**
V8	**	***	***
R2	ns	***	ns
R3	ns	**	ns
R4	**	***	**
early R5	***	***	***
late R5	***	***	***
R6	***	***	***

Shoot nitrogen concentration

	Source		
Stage	Water treatment (wtrt)	Genotype	wtrt x genotype
V5	ns	***	**
V7	**	***	*
V8	**	**	ns
R2	ns	**	ns
R3	*	**	ns
R4	ns	ns	ns
early R5	*	*	ns
late R5	**	***	ns
R6	***	**	ns

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table C. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect (Ur_CIM_{ww}) in Fayetteville 2011. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	Ur_CIM_{ww}	Water trt	Ur_CIM_{ww} x wtrt	Ur_CIM_{ww} x Ur_CIM_{ww}	Ur_CIM_{ww} x Ur_CIM_{ww} x wtrt
V5	ns	ns	ns	ns	ns
V7	***	ns	ns	**	ns
V8	ns	***	ns	ns	ns
R2	ns	ns	ns	ns	ns
R3	ns	ns	ns	ns	ns
R4	ns	**	ns	ns	ns
early R5	ns	***	ns	ns	ns
late R5	ns	***	ns	ns	ns
R6	ns	***	ns	ns	ns

Ns= not significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table D. Regression analysis of shoot ureide concentration under drought conditions versus ureide cumulative additive effect (Ur_CIM_{dr}) in Fayetteville 2011.

	Source of variation	
Stage	Ur_CIM_{dr}	Ur_CIM_{dr} x Ur_CIM_{dr}
V5	*	ns
V7	ns	ns
V8	**	ns
R2	**	ns
R3	ns	**
R4	**	ns
early R5	ns	ns
late R5	ns	ns
R6	ns	ns

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table E. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect detected by multiple interval mapping under well-watered conditions (Ur_MIM_{ww}) in Fayetteville 2011. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	Ur_MIM_{ww}	Water trt	Ur_MIM_{ww} x wtrt	Ur_MIM_{ww} x Ur_MIM_{ww}	Ur_MIM_{ww} x Ur_MIM_{ww} x wtrt
V5	ns	**	ns	ns	ns
V7	**	ns	ns	ns	*
V8	ns	**	ns	ns	ns
R2	ns	ns	ns	ns	ns
R3	ns	ns	ns	**	ns
R4	ns	**	ns	*	ns
early R5	ns	***	**	**	ns
late R5	ns	***	**	**	*
R6	*	***	**	**	*

Water trt = water treatment.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table F. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect (N_CIM_{ww}) in Fayetteville 2011. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	N_CIM_{ww}	Wtrt	N_CIM_{ww} x wtrt	N_CIM_{ww} x N_CIM_{ww}	N_CIM_{ww} x N_CIM_{ww} x wtrt
V5	**	ns	ns	ns	ns
V7	ns	*	ns	ns	ns
V8	ns	**	ns	ns	ns
R2	ns	*	ns	ns	ns
R3	ns	*	ns	ns	ns
R4	ns	ns	ns	ns	ns
early R5	ns	**	*	ns	ns
late R5	ns	***	ns	ns	ns
R6	ns	***	ns	ns	ns

Wtrt = water treatment.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table G. Regression analysis of shoot nitrogen concentration under drought conditions versus nitrogen cumulative additive effect (N_CIM_{dr}) in Fayetteville 2011.

	Source of variation	
Stage	N_CIM_{dr}	N_CIM_{dr} x N_CIM_{dr}
V5	ns	ns
V7	**	**
V8	ns	ns
R2	ns	ns
R3	ns	ns
R4	ns	ns
early R5	ns	ns
late R5	ns	ns
R6	ns	ns

Ns= not significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

Appendix Table H. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect (N_MIM_{ww}) in Fayetteville 2011. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation		
Stage	N_MIM_{ww}	wtrt	N_MIM_{ww} x wtrt
V5	***	ns	ns
V7	**	**	ns
V8	ns	***	ns
R2	*	*	ns
R3	**	ns	ns
R4	ns	ns	ns
early R5	ns	***	ns
late R5	ns	***	ns
R6	ns	***	ns

Wtrt= water treatment.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table I. ANOVA table for nitrogen fixation rate (mg N m^{-2} day^{-1}) in Fayetteville 2011.

Source	DF	MS	F Value	Pr>F
Water treatment	1	288633	974.35	<0.0001
Genotype	11	5214	1.89	0.06
Water treatment x genotype	11	1216	0.44	0.93

Appendix Table J. ANOVA table for grain yield (g m^{-2}) in Fayetteville 2011.

Source	DF	MS	F Value	Pr>F
Water treatment	1	386207	122.38	0.0016
Genotype	11	3013	1.07	0.39
Water treatment x genotype	11	3398	1.21	0.29

Appendix Table K. Analysis of variance (ANOVA) for shoot ureide concentration and nitrogen concentration in Fayetteville 2012.

Shoot ureide concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	585.47	10.98	0.016
Stage	3	4290.2	394.01	<0.0001
Genotype	17	296.11	21.8	<0.0001
Water treatment x stage x genotype	50	15.45	1.42	0.0426

Shoot nitrogen concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	1.911	18.74	0.0049
Stage	3	44.08	67.86	<0.0001
Genotype	17	0.44	4.4	<0.0001
Water treatment x stage	3	0.31	4.68	0.0033
Water treatment x genotype	17	0.157	1.58	0.082
Stage x genotype	50	0.074	1.11	0.28
Water treatment x stage x genotype	50	0.059	0.89	0.6844

Appendix Table L. Analysis of variance by phenological stage for shoot ureide and nitrogen concentration in Fayetteville 2012.

Shoot ureide concentration

	Source		
Stage	Water treatment (wtrt)	Genotype	wtrt x genotype
V6†		ns	
R2	**	***	ns
R4	**	***	**
R5	ns	***	ns

Shoot nitrogen concentration

	Source
Stage	Water treatment (wtrt)
V6†	
R2	*
R4	**
R5	ns

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

† In Fayetteville 2012 water treatment was initiated at R2 developmental stage. Consequently, at V6 only samples from the WW treatment were collected.

Appendix Table M. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect (Ur_CIM_{ww}) in Fayetteville 2012. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	Ur_CIM_{ww}	wtrt	Ur_CIM_{ww} x wtrt	Ur_CIM_{ww} x Ur_CIM_{ww}	Ur_CIM_{ww} x Ur_CIM_{ww} x wtrt
V6†	ns			ns	
R2	***	**	ns	***	ns
R4	***	*	ns	*	ns
R5	***	ns	ns	*	ns

† In Fayetteville 2012 water treatment was initiated at R2 developmental stage. Consequently, at V6 only samples from the WW treatment were collected.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table N. Regression analysis of shoot ureide concentration under drought conditions versus ureide cumulative additive effect (Ur_CIM_{dr}) in Fayetteville 2012.

	Source of variation	
Stage	Ur_CIM_{dr}	Ur_CIM_{dr} x Ur_CIM_{dr}
R2	ns	ns
R4	ns	ns
R5	ns	ns

Ns= not significant at the 0.05 probability level.

Appendix Table O. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect detected by multiple interval mapping under well-watered conditions (Ur_MIM_{ww}) in Fayetteville 2012. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	Ur_MIM_{ww}	wtrt	Ur_MIM_{ww} x wtrt	Ur_MIM_{ww} x Ur_MIM_{ww}	Ur_MIM_{ww} x Ur_MIM_{ww} x wtrt
V6†	ns			ns	
R2	***	***	ns	**	ns
R4	**	**	ns	*	ns
R5	***	ns	ns	ns	ns

† In Fayetteville 2012 water treatment was initiated at R2 developmental stage. Consequently, at V6 only samples from the WW treatment were collected.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table P. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect (N_CIM_{ww}) in Fayetteville 2012. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation				
Stage	N_CIM_{ww}	wtrt	N_CIM_{ww} x wtrt	N_CIM_{ww} x N_CIM_{ww}	N_CIM_{ww} x N_CIM_{ww} x wtrt
V6†	ns			ns	
R2	***	*	ns	ns	ns
R4	ns	**	*	**	ns
R5	**	ns	ns	*	ns

† In Fayetteville 2012 water treatment was initiated at R2 developmental stage. Consequently, at V6 only samples from the WW treatment were collected.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table Q. Regression analysis of shoot nitrogen concentration under drought conditions versus nitrogen cumulative additive effect (N_CIM_{dr}) in Fayetteville 2012.

	Source of variation	
Stage	N_CIM_{dr}	N_CIM_{dr} x N_CIM_{dr}
R2	**	ns
R4	ns	ns
R5	ns	ns

** Significant at the 0.01 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table R. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect detected by multiple interval mapping under well-watered conditions (N_MIM_{ww}) in Fayetteville 2012. When significant, water treatment (wtrt) was used as a covariate in this analysis, otherwise analysis was done using linear regression.

	Source of variation		
Stage	N_MIM_{ww}	wtrt	N_MIM_{ww} x wtrt
V6†	ns		
R2	**	**	ns
R4	ns	***	ns
R5	**	*	ns

† In Fayetteville 2012 water treatment was initiated at R2 developmental stage. Consequently, at V6 only samples from the WW treatment were collected.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table S. ANOVA table for nitrogen fixation rate (mg N m^{-2} day^{-1}) in Fayetteville 2012.

Source	DF	MS	F Value	Pr>F
Water treatment	1	60188	3.61	0.15
Genotype	16	15411	2.63	0.0036
Water treatment x genotype	16	7215	1.23	0.27

Appendix Table T. ANOVA table for grain yield (g m^{-2}) in Fayetteville 2012.

Source	DF	MS	F Value	Pr>F
Water treatment	1	50239	2.52	0.21
Genotype	17	7226	3.81	<0.0001
Water treatment x genotype	17	2083	1.1	0.37

Appendix Table U. Analysis of covariance for nitrogen fixation (mg N m^{-2} day^{-1}) and yield (g m^{-2}) versus ureide cumulative additive effects for well-watered conditions detected by composite interval mapping (Ur_CIM_{ww}) in Fayetteville 2012. Water treatment was used as a covariate in this analysis.

Source of variation	N_2 fixation	Yield
Water treatment (wtrt)	**	***
Ur_CIM_{ww}	ns	***
Ur_CIM_{ww} x wtrt	**	*

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table V. Analysis of covariance for nitrogen fixation (mg N m^{-2} day^{-1}) and yield (g m^{-2}) versus ureide cumulative additive effects for well-watered conditions detected by multiple interval mapping (Ur_MIM_{ww}) in Fayetteville 2012. Water treatment was used as a covariate in this analysis.

Source of variation	N_2 fixation	Yield
Water treatment (wtrt)	**	***
Ur_MIM_{ww}	ns	***
Ur_MIM_{ww} x wtrt	**	***

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table W. Analysis of covariance for nitrogen fixation (mg N m^{-2} day^{-1}) and yield (g m^{-2}) versus nitrogen cumulative additive effects for well-watered conditions detected by composite interval mapping (N_CIM_{ww}) in Fayetteville 2012. Water treatment was used as a covariate in this analysis.

Source of variation	N_2 fixation	Yield
Water treatment (wtrt)	**	***
N_CIM_{ww}	ns	**
N_CIM_{ww} x wtrt	*	*

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table X. Analysis of variance (ANOVA) for shoot ureide concentration and nitrogen concentration in Keiser 2012. Drought treatment was included at R2 only. Data from other developmental stages was collected under well-watered conditions.

Shoot ureide concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	22.4	1.4	0.28
Stage	2	16510.93	1752.68	<0.0001
Genotype	16	140	19.52	<0.0001
Stage x genotype	32	103.3	10.97	<0.0001
Water treatment x genotype	16	7.13	0.99	0.47

Shoot nitrogen concentration

Source	DF	MS	F Value	Pr>F
Water treatment	1	0.0953	1.93	0.21
Stage	2	6.307	249.5	<0.0001
Genotype	16	0.302	7.13	<0.0001
Stage x genotype	32	0.087	3.44	<0.0001
Water treatment x genotype	16	0.0838	1.98	0.025

Appendix Table Y. Ureide and nitrogen concentration in the WW and DR treatment at R2 developmental stage in Keiser 2012.

Water treatment	Ureide (μmol g^{-1})	Nitrogen (%N)
WW	18.2a	2.61a
DR	18.9a	2.57a

Means followed by the same letter were determined to be statistically no different by an F-test (*P*=0.05).

Appendix Table Z. Regression analysis of shoot ureide concentration under drought conditions versus ureide cumulative additive effect (Ur_CIM_{dr}) in Keiser 2012 at R2 developmental stage.

Source of variation	
Ur_CIM_{dr}	ns
Ur_CIM_{dr} x Ur_CIM_{dr}	ns

Ns= not significant at the 0.05 probability level.

Appendix Table AA. Regression analysis of shoot nitrogen concentration under drought conditions versus nitrogen cumulative additive effect (N_CIM_{dr}) in Keiser 2012 at R2 developmental stage.

Source of variation	
N_CIM_{dr}	**
N_CIM_{dr} x N_CIM_{dr}	ns

** Significant at the 0.01 probability level.

Ns= not significant at the 0.05 probability level.

Appendix Table AB. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect (Ur_CIM_{ww}) in Keiser 2012 at R2 developmental stage. Water treatment was used as a covariate. At V6 and R4 developmental stages, analysis was done using linear regression of shoot ureide concentration versus Ur_CIM_{ww}.

	Source of variation				
Stage	Ur_CIM_{ww}	Water trt	Ur_CIM_{ww} x wtrt	Ur_CIM_{ww} x Ur_CIM_{ww}	Ur_CIM_{ww} x Ur_CIM_{ww} x wtrt
V6†	ns			**	
R2	ns	ns	ns	***	ns
R4†	***			*	

† There was no drought treatment at V6 and R4 stages in Keiser 2012. Data from these stages were collected under well-watered conditions.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AC. Analysis of covariance for shoot ureide concentration versus ureide cumulative additive effect detected by multiple interval mapping under well-watered conditions (Ur_MIM_{ww}) in 2011 Keiser 2012 at R2 developmental stage. Water treatment (wtrt) was used as a covariate in this analysis. At V6 and R4 developmental stages, analysis was done using linear regression of shoot ureide concentration versus Ur_MIM_{ww}.

	Source of variation				
Stage	Ur_MIM_{ww}	wtrt	Ur_MIM_{ww} x wtrt	Ur_MIM_{ww} x Ur_MIM_{ww}	Ur_MIM_{ww} x Ur_MIM_{ww} x wtrt
V6†	ns			**	
R2	*	ns	ns	***	ns
R4†	**			ns	

† There was no drought treatment at V6 and R4 stages in Keiser 2012. Data from these stages were collected under well-watered conditions.

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

** Significant at the 0.01 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AD. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect (N_CIM_{ww}) in Keiser 2012 at R2 developmental stage. Water treatment (wtrt) was used as a covariate in this analysis. At V6 and R4 developmental stages, analysis was done using linear regression of shoot ureide concentration versus N_CIM_{ww}.

	Source of variation				
Stage	N_CIM_{ww}	wtrt	N_CIM_{ww} x wtrt	N_CIM_{ww} x N_CIM_{ww}	N_CIM_{ww} x N_CIM_{ww} x wtrt
V6†	ns			ns	
R2	***	ns	ns	ns	ns
R4†	***			ns	

† There was no drought treatment at V6 and R4 stages in Keiser 2012. Data from these stages were considered under well-watered conditions.

Ns= not significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AE. Analysis of covariance for shoot nitrogen concentration versus nitrogen cumulative additive effect (N_MIM_{ww}) in Keiser 2012 at R2 developmental stage. Water treatment (wtrt) was used as a covariate in this analysis. At V6 and R4 developmental stages, analysis was done using linear regression of shoot ureide concentration versus N_MIM_{ww}.

	Source of variation		
Stage	N_MIM_{ww}	wtrt	N_MIM_{ww} x wtrt
V6†	*		
R2	***	ns	ns
R4†	***		

† There was no drought treatment at V6 and R4 stages in Keiser 2012. Data from these stages were considered under well-watered conditions.

*Significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.

Ns= not significant at the 0.05 probability level.

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Appendix Table AF. ANOVA table for percentage of nitrogen derived from the atmosphere (%Ndfa) in Keiser 2012 at R2 and R4 developmental stages.

Source	DF	MS	F Value	Pr>F
Water treatment	1	265.6	2.97	0.1
Genotype	16	37.2	2.33	0.004
Stage	1	2903.1	182.13	<0.0001
Water treatment x genotype	16	31.4	1.97	0.02
Stage x genotype	16	9.7	0.61	0.9

† There was no drought treatment at R4 stages in Keiser 2012. Data from these stages were considered under well-watered conditions.

Appendix Table AG. Analysis of covariance for percentage of nitrogen derived from the atmosphere (%Ndfa) versus ureide cumulative additive effects detected for well-watered conditions by multiple interval mapping (Ur_MIM_{ww}) in Keiser 2012. Developmental stage was used as a covariate in this analysis.

Source of variation	Significance
Water treatment (wtrt)	ns
Stage	***
Ur_MIM_{ww}	ns
Ur_MIM_{ww} x wtrt	ns
Ur_MIM_{ww} x stage	ns
Ur_MIM_{ww} x Ur_MIM_{ww}	ns

Ns= not significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AH. Analysis of covariance for percentage of nitrogen derived from the atmosphere (%Ndfa) versus nitrogen cumulative additive effects detected for well-watered conditions by multiple interval mapping (N_MIM_{ww}) in Keiser 2012. Developmental stage was used as a covariate in this analysis.

Source of variation	Significance
Water treatment (wtrt)	ns
Stage	***
N_MIM_{ww}	ns
N_MIM_{ww} x wtrt	ns
N_MIM_{ww} x stage	ns
N_MIM_{ww} x N_MIM_{ww}	ns

Ns= not significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AI. Analysis of covariance for percentage of nitrogen derived from the atmosphere (%Ndfa) versus ureide cumulative additive effects detected for well-watered conditions by composite interval mapping (Ur_CIM_{ww}) in Keiser 2012. Developmental stage was used as a covariate in this analysis.

Source of variation	Significance
Water treatment (wtrt)	ns
Stage	***
Ur_CIM_{ww}	*
Ur_CIM_{ww} x wtrt	ns
Ur_CIM_{ww} x stage	ns
Ur_CIM_{ww} x Ur_CIM_{ww}	ns

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.

Appendix Table AJ. Analysis of covariance for percentage of nitrogen derived from the atmosphere (%Ndfa) versus nitrogen cumulative additive effects detected for well-watered conditions by composite interval mapping (N_CIM_{ww}) in Keiser 2012. Developmental stage was used as a covariate in this analysis.

Source of variation	Significance
Water treatment (wtrt)	ns
Stage	***
N_CIM_{ww}	*
N_CIM_{ww} x stage	ns
N_CIM_{ww} x wtrt	ns
N_CIM_{ww} x N_CIM_{ww}	*
N_CIM_{ww} x N_CIM_{ww} x stage	ns
N_CIM_{ww} x N_CIM_{ww} x wtrt	ns

Ns= not significant at the 0.05 probability level.

*Significant at the 0.05 probability level.

*** Significant at the 0.0001 probability level.